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(54) Title: rAAV-VECTORED RIBOZYME COMPOSITIONS AND METHODS FOR THE TREATMENT OF RETINAL DISEASES

(57) Abstract: Disclosed are ribozymes, as well as compositions, vectors, virus particles, host cells, and therapeutic kits that comprise them, which have been shown to be useful in the manufacture of medicaments and in the treatment of diseases of the mammalian eye, and in particular retinopathy and macular degeneration. Also disclosed are methods and uses of these compositions in the reduction, elimination, and/or amelioration of symptoms of diseases of the human eye, including for example, loss of vision, retinitis, retinopathy, and blindness.

DESCRIPTION

RAAV-VECTORED RIBOZYME COMPOSITIONS AND METHODS FOR THE TREATMENT OF RETINAL DISEASES

1. BACKGROUND OF THE INVENTION

The present application is a continuation-in-part of co-pending U. S. application Serial No. 09/847,601 filed May 1, 2001, which was a continuation-in-part of application Serial No. 09/063,667, filed April 21, 1998, now issued as U. S. Patent Number 6,225,291, which claimed priority from provisional application Serial Nos. 60/046,147 and 60/044,492, filed May 9, 1997 and April 21, 1997, respectively, each now abandoned. The entire contents of each are specifically incorporated herein by reference in their entirety. The United States government has certain rights in the present invention pursuant to grant number EY08571 from the National Institutes of Health.

1.1 FIELD OF THE INVENTION

The present invention relates generally to the fields of genetics, molecular and cellular biology, and medicine. More particularly, it concerns ribozymes, as well as AAV-based vectors, virus, host cells, and kits comprising them, as well as methods for their use in treating or reducing the severity or symptoms of a variety of diseases of the mammalian eye, including, for example, retinal degeneration, retinitis pigmentosa, macular degeneration, and retinopathy.

1.2 DESCRIPTION OF RELATED ART

1.2.1 RIBOZYMES

Ribozymes are biological catalysts consisting of only RNA. They promote a variety of reactions involving RNA and DNA molecules including site-specific cleavage, ligation, polymerization, and phosphoryl exchange (Cech, 1989; Cech, 1990). Ribozymes fall into three broad classes: (1) RNAse P, (2) self-splicing introns, and (3) self-cleaving viral agents. Self-cleaving agents include hepatitis delta virus and components of plant virus satellite RNAs that sever the RNA genome as part of a rolling-circle mode of replication. Because of their small size and great specificity, ribozymes have the greatest potential for biotechnical applications. The ability of ribozymes to cleave other RNA molecules at specific sites in a catalytic manner

has brought them into consideration as inhibitors of viral replication or of cell proliferation and gives them potential advantage over antisense RNA. Indeed, ribozymes have already been used to cleave viral targets and oncogene products in living cells (Koizumi *et al.*, 1992; Kashani-Sabet *et al.*, 1992; Taylor and Rossi, 1991; von-Weizsacker *et al.*, 1992; Ojwang *et al.*, 1992; Stephenson and Gibson, 1991; Yu *et al.*, 1993; Xing and Whitton, 1993; Yu *et al.*, 1995; Little and Lee, 1995).

Two kinds of ribozymes have been employed widely, hairpins and hammerheads. Both catalyze sequence-specific cleavage resulting in products with a 5N hydroxyl and a 2N,3N-cyclic phosphate. Hammerhead ribozymes have been used more commonly, because they impose few restrictions on the target site. Hairpin ribozymes are more stable and, consequently, function better than hammerheads at physiologic temperature and magnesium concentrations.

A number of patents have issued describing various ribozymes and methods for designing ribozymes. See, for example, U.S. Patent Nos. 5,646,031; 5,646,020; 5,639,655; 5,093,246; 4,987,071; 5,116,742; and 5,037,746, each specifically incorporated herein by reference in its entirety. However, the ability of ribozymes to provide therapeutic benefit *in vivo* has not yet been demonstrated.

1.2.2 DISEASES OF THE EYE

There are more than 200 inherited diseases that lead to retinal degeneration in humans. Considerable progress has been made in identifying genes and mutations causing many forms of inherited retinal degeneration in humans and other animals. Diseases causing inherited retinal degeneration in humans can be classified broadly into those that first affect peripheral vision and the peripheral retina, such as retinitis pigmentosa, and those that primarily affect central vision and the macula, such as macular dystrophy. The macula has the highest concentration of cones and the peripheral retina is dominated by rods.

Retinitis pigmentosa (RP) is a collection of heritable retinal degenerations caused by defects in one of several genes for proteins of photoreceptor (PR) cells. RP is characterized by progressive rod photoreceptor degeneration and eventual blindness. The exact molecular pathogenesis of RP is still unexplained. Ultrastructural observations suggest that the rod PRs are severely affected in the disease. Approximately 50,000 individuals in the United States are estimated to have RP. The clinical symptoms of retinitis pigmentosa include night blindness and loss of peripheral vision. With time, visual impairment progresses toward the center of the retina causing "tunnel-vision."

Retinitis pigmentosa can be subdivided into several genetic categories: antosomal dominant (adRP), autosomal recessive (arRP), X-linked (xIRP) or syndromic. There are also a number of clinical classes for retinitis pigmentosa. These classes have been condensed into two broad categories. Type 1 retinitis pigmentosa is characterized by rapid progression and diffuse, severe pigmentation; type 2 retinitis pigmentosa has a slower progression and more regional, less severe pigmentation.

Macular degeneration is a deterioration of the macula (the cone-rich center of vision) leading to gradual loss of central vision. Eventual loss of these cones leads to central vision loss and functional blindness. At least 500,000 individuals are estimated to suffer from macular degeneration currently in the United States. Macular degeneration can have either a genetic basis or it may be an acquired disease. Approximately 10% of Americans over the age of 50 are afflicted with age-related macular degeneration, an acquired form of disease. The inherited forms of macular degeneration are much less common but usually more severe. Inherited macular degeneration is characterized by early development of macular abnormalities such as yellowish deposits and atrophic or pigmented lesions, followed by progressive loss of central vision.

There are many other inherited diseases that also cause retinal degeneration in humans. Among these are gyrate atrophy, Norrie disease, choroideremia and various cone-rod dystrophies. In addition there are numerous inherited systemic diseases, such as Bardet-Biedl, Charcot-Marie-Tooth, and Refsum disease which include retinal degeneration among a multiplicity of other symptoms.

Another important ocular disease is diabetic retinopathy, the leading cause of blindness in adults between the ages of 18 and 72. Histological studies consistently implicate endothelial cell dysfunction in the pathology. A hallmark of advancing diabetic retinopathy is aberrant retinal neovascularization, termed proliferative diabetic retinopathy (PDR).

Hyperglycemia directly contributes to the development of diabetic retinopathy, and early in the development of diabetic retinopathy there exists disruption of the blood-retinal barrier. NOS activity, as determined by conversion of arginine to citrulline, is significantly increased in diabetes Rosen *et al.*, 1995). Gade and coworkers demonstrated that endothelial cell dysfunction correlated with elevated glucose in an *in vitro* wound model and was mediated by increased levels of NO (Gade *et al.*, 1997). In rat cerebral arteries acute glucose exposure dilates arteries via an endothelium mediated mechanism that involves NO (Cipolla *et al.*, 1997). Cosentino demonstrated that prolonged exposure to high glucose increases eNOS gene expression, protein synthesis, and NO release (Cosentino *et al.*, 1997).

Nitric oxide (NO) is a pleiotropic molecule with multiple physiological effects: neurotransmitter, component of the immune defense system, regulator of smooth muscle tone and blood pressure, inhibitor of platelet aggregation and a superoxide scavenger. NO is synthesized as a product of the conversion of L-arginine into L-citrulline by the so-called constitutive nitric oxide synthase (NOS), either neuronal (nNOS) or endothelial (eNOS) isoforms. NO regulates specific protein levels. NO increases mRNA levels for VEGF and iNOS.

Although several studies on NO function in the retina have been published, very little information is available pertaining to its role in the diabetic retina (Chakravarthy *et al.*, 1995; Goldstein *et al.*, 1996). The iNOS isoform is expressed in the retina, as shown by RT-PCR™ and immunocytochemistry. It is believed to be involved in the development of diabetic retinopathy and in ischemia-reperfusion injury (Hangai *et al.*, 1996; Ostwald *et al.*, 1995). Administering NOS inhibitors can ameliorate or prevent ischemia-reperfusion injury (Lam and Tso, 1996). Diabetic human retinal pigmented epithelial cells have augmented iNOS compared to non-diabetic cells. An increasing body of evidence indicates growth factors including vascular endothelial growth factor (VEGF) and insulin-like growth factor-I (IGF-I) are involved in increased permeability of endothelium that leads to breakdown of the blood-retinal barrier in this microvascular disease. However, the mechanisms for growth factor action in disease progression remain elusive.

1.2.3 Deficiencies in the Prior Art

There is currently no effective treatment for most forms of retinitis pigmentosa or macular degeneration. Treatment with a massive supplement (15,000 I.U. per day) of vitamin A often retards the course of retinal degeneration in retinitis pigmentosa. Vitamin therapy does not treat the underlying cause of RP, and is not a cure.

Also what are lacking are feasible approaches for the systemic or local administration of retinal therapeutic agents that can halt or prevent damage from retinal diseases, including for example, neovascularization in patients with diabetic retinopathy. Although considerable attention has been given to vascular endothelial growth factor (VEGF), an increasing body of evidence implicates insulin-like growth factor-I (IGF-I) in the pathogenesis of aberrant neovascularization that characterizes PDR (Frank, 1990; Smith *et al.*, 1997). It has been demonstrated that the adenosine A_{2B} receptor is expressed in angiogenic blood vessels, and that activation of this receptor results in local VEGF and IGF-I production. Adenosine acting through A_{2B} receptors links altered cellular metabolism caused by oxygen deprivation to

compensatory angiogenesis. Adaptation to hypoxia includes induction of diverse genes that appear to depend on a common mode of oxygen sensing and signal transduction, triggering the activation of critical transcription factors, hypoxia-inducible factors (HIFs). The development of targeted gene therapy methods to address such limitations in the art, and to develop therapeutic compositions to effectively treat diseases of the mammalian retina would represent a significant advancement in the fields of medicine and, in particular, ophthalmology and the treatment of disorders and diseases of the human eye.

2.0 SUMMARY OF THE INVENTION

The present invention overcomes these and other inherent limitations in the prior art, by providing materials and methods for the treatment of diseases of the mammalian eye. More specifically, the subject invention provides polynucleotide sequences, and methods for using these sequences, to achieve highly specific degradation or reduction of mRNAs encoding polypeptides that cause, contribute to, or participate in disease and dysfunction of the eye, and in particular, the retina. As described herein, the materials and methods of the subject invention can be used to treat a variety of ophthalmic disorers and diseases. In preferred embodiments, the invention provides compositions, methods, and therapeutic kits for treatment and/or the amelioration of symptoms of diseases and disorders of the human eye, such as for example, retinitis, retinitis pigmentosa (RP), autosomal dominant retinitis pigmentosa (ADRP), retinopathy, diabetic retinopathy, macular degeneration, age-related macular degeneration, and a variety of related disorders.

In particular illustrative embodiments described herein, the subject invention employs the use of novel catalytic ribonucleotide compounds, and in particular, hammerhead and/or hairpin ribozymes, that have been designed to cleave mutant forms of messenger RNA (mRNA) occurring in various forms of ocular diseases and retinal damage or degeneration. These ribozyme compounds have been designed and particularly selected such that the catalytic domain of each ribozyme has highly effective, stable, selective activity in cleaving target mRNAs to bring about a reduction in, or an elimination of, the encoded polypeptide produced from translation of the mRNA by cellular protein synthesis machinery.

In a first embodiment, the present invention provides a ribozyme that specifically cleaves an mRNA encoding a polypeptide that causes or contributes to the disease, disorder, or dysfunction of a cell or a tissue of a mammalian eye.

Preferred ribozymes include those catalytic RNA molecules, that specifically cleave an mRNA encoding a polypeptide selected from the group consisting of rod opsin, RP1,

RDS/Peripherin, iNOS, Adenosine A_{2B} , Adenosine A_{2B} Receptor, IGF-1, IGF-1 Receptor (IGF-1R), VEGF, VEGF receptor, integrin alpha 1, integrin alpha 3, integrin alpha 5, and integrin alpha V. Exemplary ribozymes of the present invention include, but are not limited to, those that catalytic RNA molecules that comprise, consist essentially of, or consist of, the nucleotide sequence of any one of SEQ ID NO:2 and SEQ ID NO:90 to SEQ ID NO:114. As such, the present invention encompasses catalytic RNA molecules that specifically cleave one or more mRNAs that comprise at least a first ribozyme-specific target sequence, wherein the target sequence comprises, consists essentially of, or consists of, a sequence selected from any one of SEQ ID NO:3 to SEQ ID NO:89, SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107, SEQ ID NO:108, SEQ ID NO:109, and SEQ ID NO:110.

Exemplary ribozymes of the invention are shown in SEQ ID NO:2, SEQ ID NO:90, SEQ ID NO:91, SEQ ID NO:92, SEQ ID NO:93, SEQ ID NO:94, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:100, SEQ ID NO:101, SEQ ID NO:102, SEQ ID NO:103, SEQ ID NO:104, SEQ ID NO:111, SEQ ID NO:112, SEQ ID NO:113, and SEQ ID NO:114.

Preferred ribozymes of the invention encompass those catalytic RNA molecules that specifically cleave an mRNA that encodes a polypeptide selected from the group consisting of a mutant rod opsin polypeptide, a mutant RP1 polypeptide, a mutant RDS/Peripherin polypeptide, a mutant iNOS polypeptide, a mutant adenosine A_{2B} receptor polypeptide, a mutant adenosine A_3 receptor polypeptide, a mutant IGF-1 polypeptide, a mutant IGF-1R polypeptide, a mutant integrin alpha 1 polypeptide, a mutant integrin alpha 3 polypeptide, and a mutant integrin alpha V polypeptide.

Exemplary ribozymes preferred in the practice of the invention include those that specifically cleave an mRNA encoding a mutant rod opsin polypeptide that comprises a mutation selected from the group consisting of P23H, P23L, Q28H, F45L, L46R, G51A, G51G, G51R, G51V, P53R, T58R, Q64stop, 68-71, V87D, G90D, G106W, C110Y, G114D, R135G, R135L, R135P, P171L, P171S, Y178C, P180A, C187Y, G188R, D190G, D190Y, M207R, H211R, H211P, F220C, C264X, P267L, F220C, C222R, A292E, Q344stop, and P347S. Such designations follow the standard protein nomenclature, in that a "P23H" mutation is one in which the native amino acid at position 23 of the polypeptide (in this case Pro) is changed *via* mutagenesis to a His. Likewise, an F200C mutant is a peptide where the Phe at position 200 is changed to a Cys residue at that position, and so forth.

Exemplary ribozymes of the present invention include, but are not limited to, those catalytic RNA molecules that specifically cleave one or more rod opsin-sepcific mRNAs that

comprise, consist essentially of, or consist of, a nucleotide sequence that comprises, consists essentially of, or consists of, at least a first contiguous nucleotide sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, SEQ ID NO:62, SEQ ID NO:63, SEQ ID NO:78, SEQ ID NO:79, and SEQ ID NO:86. Particularly preferred rod opsin-specific ribozymes include, but are not limited to, those that comprise, consist essentially of, or consist of, the nucleotide sequence of SEQ ID NO:102 or SEQ ID NO:103.

Exemplary ribozymes of the present invention also include, but are not limited to, those catalytic RNA molecules that specifically cleave one or more mRNAs encoding a mutant RP1 polypeptide. Such ribozymes include those that specifically cleaves an RP1-specific mRNA that comprises a target sequence that comprises, consists essentially of, or consists of, the nucleotide sequence of SEQ ID NO:64.

Exemplary ribozymes of the present invention also include, but are not limited to, those catalytic RNA molecules that specifically cleave one or more mRNAs encoding a mutant RDS/Peripherin polypeptide. Such ribozymes include those that specifically cleave an mRNA that comprises a target sequence that comprises, consists essentially of, or consists of a nucleotide sequence selected from the group consisting of SEQ ID NO:65, SEQ ID NO:66, SEQ ID NO:67, SEQ ID NO:68, SEQ ID NO:69, SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO:73, SEQ ID NO:74, SEQ ID NO:75, SEQ ID NO:76, and SEQ ID NO:77. These include, but are not limited to, mRNAs that comprise a mutation in RDS/Peripherin that is selected from the group consisting of the C118, R172Q, R172W, P210R, C214S, P216L, and P219 mutations.

Exemplary ribozymes of the present invention also include, but are not limited to, those catalytic RNA molecules that specifically cleave one or more mRNAs encoding a

mutant Adenosine A_{2B} Receptor polypeptide, for example, those ribozymes that comprise the sequence of SEQ ID NO:90 or SEQ ID NO:91. Such ribozymes include, but are not limited to, catalytic RNA molecules that specifically cleave those mRNAs comprising a target sequence that comprises, consists essentially of, or consists of the nucleotide sequence of SEQ ID NO:80.

Exemplary ribozymes of the present invention also include, but are not limited to, those catalytic RNA molecules that specifically cleave one or more mRNAs encoding a mutant iNOS polypeptide, for example, those ribozymes that comprise the sequence of SEQ ID NO:104. Such ribozymes include, but are not limited to, catalytic RNA molecules that specifically cleave those mRNAs comprising a target sequence that comprises, consists essentially of, or consists of the nucleotide sequence of SEQ ID NO:81.

Exemplary ribozymes of the present invention also include, but are not limited to, those catalytic RNA molecules that specifically cleave one or more mRNAs encoding a mutant integrin alpha 1 polypeptide, for example, those ribozymes that comprise the sequence of SEQ ID NO:96 or SEQ ID NO:97. Such ribozymes include, but are not limited to, catalytic RNA molecules that specifically cleave those mRNAs comprising a target sequence that comprises, consists essentially of, or consists of, the nucleotide sequence of SEQ ID NO:82 or SEQ ID NO:83.

Exemplary ribozymes of the present invention also include, but are not limited to, those catalytic RNA molecules that specifically cleave one or more mRNAs encoding a mutant integrin alpha 5 polypeptide, for example, those ribozymes that comprise the sequence of SEQ ID NO:94 or SEQ ID NO:95. Such ribozymes include, but are not limited to, catalytic RNA molecules that specifically cleave those mRNAs comprising a target sequence that comprises, consists essentially of, or consists of, the nucleotide sequence of SEQ ID NO:84, SEQ ID NO:85, or SEQ ID NO:106.

Exemplary ribozymes of the present invention also include, but are not limited to, those catalytic RNA molecules that specifically cleave one or more mRNAs encoding a mutant integrin alpha 3 polypeptide, for example, those ribozymes that comprise the sequence of SEQ ID NO:92 or SEQ ID NO:93. Such ribozymes include, but are not limited to, catalytic RNA molecules that specifically cleave those mRNAs comprising a target sequence that comprises, consists essentially of, or consists of, the nucleotide sequence of SEQ ID NO:86 or SEQ ID NO:87.

Exemplary ribozymes of the present invention also include, but are not limited to, those catalytic RNA molecules that specifically cleave one or more mRNAs encoding a

mutant IGF1 receptor polypeptide, for example, those ribozymes that comprise the sequence of SEQ ID NO:100 or SEQ ID NO:101. Such ribozymes include, but are not limited to, those catalytic RNA molecules that specifically cleave those mRNAs comprising a target sequence that comprises, consists essentially of, or consists of, the nucleotide sequence of SEQ ID NO:88 or SEQ ID NO:89.

Exemplary ribozymes of the present invention also include, but are not limited to, those catalytic RNA molecules that specifically cleave one or more mRNAs encoding a mutant VEGF receptor polypeptide, for example, those ribozymes that comprise the sequence of SEQ ID NO:111, SEQ ID NO:112, SEQ ID NO:113, or SEQ ID NO:114. Such ribozymes include, but are not limited to, those catalytic RNA molecules that specifically cleave those mRNAs comprising a target sequence that comprises, consists essentially of, or consists of the nucleotide sequence of SEQ ID NO:107, SEQ ID NO:108, SEQ ID NO:109, or SEQ ID NO:110.

Exemplary ribozymes of the present invention also include, but are not limited to, those catalytic RNA molecules that specifically cleave one or more mRNAs encoding a mutant integrin alpha V polypeptide, for example, those ribozymes that comprise the sequence of SEQ ID NO:98 or SEQ ID NO:99.

As described herein, the ribozymes of the present invention preferably may be of the hairpin (FIG. 11) or the hammerhead (FIG. 12) variety.

A further aspect of the invention is a vector, virus, or host cell that comprises a polynucleotide encoding one or more such preferred ribozymes. Such vectors, virus and host cells will preferably comprise at least a first such polynucleotide that is operably linked to at least a first promoter element that directs expression of the polynucleotide in a mammalian cell to produce the desired ribozyme. Such vectors may include viral vectors such as adenoviral or adeno-associated viral vectors, and such promoter elements will preferably direct the expression of the polynucleotide in a cells and/or tissues of a mammalian, and in particular, a human eye. Exemplary host cells include retinal cells, photoreceptor cells, rod cells, cone cells, Mueller cells, and retinal pigement epithelial cells. Such vectors may include promoter element that comprise a constitutive or an inducible promoter element operable in the eye, such as, for example, a CMV promoter, a mammalian rod opsin promoter, or other suitable promoter element.

The invention also encompasses compositions, formulations, and therapeutic kits that comprise such ribozymes, vectors, virus particles, viral vectors, or host cells. These compositions preferably are formulated in pharmaceutically-acceptable excipients, suitable

for ocular or subretinal administration to a mammalian eye. The compositions may also optionally further comprise one or more carriers, adjuvants, lipids, liposomes, lipid particles, nanoparticles, or microsphere formulations to facilitate administration to the affected eye. Such kits may include one or more of the compositions of the invention along with one or more devices for administering the therapeutic agents, as well as instructions for using the kit or its components in the therapy of the eye. For example, the kits of the invention may comprise a device such as a syringe or a needle, for delivering the compositions to the eye, retina, or subretinal space of a mammal.

In another important embodiment, the invention also provides a method for decreasing the amount of mRNA encoding a selected polypeptide in a retinal cell of a mammalian eye. This method generally involves providing to the eye a ribozyme composition in an amount and for a time effective to specifically cleave the mRNA in the cell, and thereby decrease the amount of mRNA in such a cell.

Such methods find particular utility in specifically cleaving an mRNA that encodes a polypeptide that causes a pathological condition in, or contributes to a disease, disorder, or dysfunction in a cell or a tissue of a mammalian eye. Examples of such conditions include, but are not limited to, retinal degeneration, retinitis, macular degeneration, and retinopathy, and particularly include conditions such as retinitis pigmentosa, autosomal dominant retinitis pigmentosa, autosomal recessive retinitis pigmentosa, macular degeneration, age-related macular degeneration, retinopathy, and diabetic retinopathy.

Likewise, the invention provides methods for decreasing the amount of a selected polypeptide in a cell or tissue of a mammalian eye. Such methods also generally involve providing or administering to an eye, a ribozyme construct of the present invention in an amount and for a time effective to specifically decrease the amount of the selected polypeptide in the cells or tissues of the eye. Similarly, the compositions of the invention may be used in methods for decreasing the amount of a selected polypeptide in the eye of a mammal suspected of having a pathological condition, and in methods for treating, decreasing the severity, or ameliorating the symptoms of a pathological condition that results from the expression of at least a first selected polypeptide in a cell or a tissue of a human eye. Examples of such symptoms include, but are not limited to, atrophic lesions of the eye, pigmented lesions of the eye, blindness, a reduction in central vision, a reduction in peripheral vision, and a reduction in total vision.

The invention also provides methods for decreasing the progression of such degenerative pathological conditions of a mammalian eye, and these methods typically

comprise providing to such an eye one or more ribozymes, vectors, or viral particles of the invention, in an amount and for a time effective to decrease the progression of such degenerative pathological conditions.

A further aspect of the subject invention pertains to the reduction and/or elimination of pathological levels of proteins involved in endothelial cell nitric oxide (NO) regulation. This aspect of the subject invention provides materials and methods for the treatment and/or prevention of diabetic retinopathy. Increased inducible nitric oxide synthase (iNOS), enhanced vascular endothelial growth factor levels, and disruption of the blood retinal barrier has been identified in the retinas of BBZ/Wor diabetic rats compared to non-diabetic age-matched controls. Additionally, endothelial NOS (eNOS) has been identified in the plasmalemmal caveolae of retinal capillary endothelium from diabetic animals, and cytological evidence indicates translocation of the caveolae from the lumenal to the ablumenal surface of the endothelium. In high glucose environments, chronically increased NO activity results in endothelial cell dysfunction and impaired blood-retinal barrier integrity responsible for the development of diabetic retinopathy.

The ribozyme compositions of the present invention are preferably comprised within a vector suitable for delivery and expression in selected cells and tissues of the mammalian eye. For example, viral delivery vectors, and AAV-based vectors and virus particles are particularly preferred for delivery of the therapeutic catalytic molecules to the affected eye and the host cells and tissues comprised within the eye. These virus-vectored ribozyme molecules can be delivered to the target site by a variety of different methods, including for example, direct injection of the pharmaceutical compositions into the eye, the subretinal space, or the tissues immediately adjacent to the affected eye. These and other aspects of the present invention will be readily apparent to those of skill in the art having benefit of the present disclosure and the specific teachings disclosed hereinbelow:

3. BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to the following description taken in conjunction with the accompanying drawings, in which like reference numerals identify like elements, and in which:

FIG. 1 shows adenosine, acting through its type A_2 receptor, can act to increase oxygen supply via two paths. During acute hypoxia, adenosine acts on smooth muscle cells, resulting in vasodilation (A_{2A}). With chronic ischemia, adenosine acts as an angiogenic agent by exerting a

mitogenic effect on microvascular endothelial cells (in HREC, A_{2B}; see below). It is this latter effect that can be interfered with in an attempt to develop a pharmacological therapy for neovascular diseases. A distinct receptor subtype that mediates solely the mitogenic effect of adenosine would allow the targeting of a selective antagonist against that receptor subtype, without preventing the vasodilation mediated by the A_{2A} receptor;

FIG. 2A shows HREC proliferation after stimulation with NECA alone or in combination with a blocking antibody to VEGF. Open bars are results after 24 hr of exposure; filled bars are results after 48 hr. (*), significantly different from 10 μM NECA alone for the respective exposure time by ANOVA ($p < 0.05$). Also shown are control cells exposed to VEGF alone or in combination with anti-VEGF to demonstrate the efficacy of the antibody;

FIG. 2B shows VEGF content in conditioned medium from HREC after stimulation with NECA in the presence or absence of sense or antisense oligonucleotides homologous to human A_{2B} adenosine receptor or to human VEGF. Assay duration was 48 hr. A_{2B} antisense treatment reduces the amount of VEGF protein secreted in response to NECA to levels equaling or exceeding the reduction evident by VEGF antisense treatment;

FIG. 3 shows NECA, at the concentrations indicated in the legends, induces a transient activation of ERK/MAPK in HREC that peaks at 5 min and desensitizes by 20 min after exposure. HREC were serum-starved for 24 hr and pre-treated for 20 min with 1 U/mL adenosine deaminase prior to adding NECA. Activated ERK/MAPK was visualized on Western blots by enhanced chemiluminescence using EC10 monoclonal antibody;

FIG. 4 shows the A_1-selective agonist CPA stimulates ERK/MAPK phosphorylation in HREC, however the A_{2A}-selective agonist CGS did not activate ERK/MAPK;

FIG. 5 shows HREC were pretreated for 30 min with the MEK inhibitor PD98059 or the PKA inhibitor H-89 and stimulated with NECA for 5 min. PD98059 inhibited ERK activation, while H-89 increased basal ERK activation. H-89 did not block NECA-stimulated ERK activation, suggesting that PKA is not involved in signaling from the adenosine receptor to ERK. The non-selective adenosine receptor antagonist XAC decreased ERK activation by high concentrations of NECA, but modestly increased ERK activation in control conditions and in response to 1 and 10 nM NECA. In contrast, PD98059 did not alter CREB, whereas both H-89 and XAC blocked NECA-induced CREB activation. These data indicate that NECA results in ERK activation independent of the cAMP response;

FIG. 6 shows both Enprofylline and JW V-108 antagonize activation of p42 and p44 ERK/MAP kinase by NECA. HRECs were serum-starved for 24 hr and pre-treated with adenosine deaminase (ADA, 1 U/mL) for 20 min, incubated with the antagonists in the presence

of ADA for 10 min. NECA (1 nM-10 μM, 10 min) was used to activate ERK. ERK activation was analyzed by Western blot using the E10 monoclonal antibody, which recognizes the phosphorylated (active) form of the enzyme;

FIG. 7 shows a schematic representation (left) of the A_{2B} adenosine receptor ribozyme shows the nucleotide sequence of the recognition arms, as well as the complementary sequence (in red) of the synthetic target. Cleavage of this target by the ribozyme is shown in the autoradiogram (top right), demonstrating the cleavage kinetics. Band densities of cleaved *vs.* intact target were plotted as percent cleaved (bottom right). The A_{2B} receptor ribozyme cleaves nearly 90% of target in a 1:1 molar ratio by 60 min;

FIG. 8 shows A_{2B} adenosine receptor ribozyme reduces NECA-stimulated VEGF synthesis and cell proliferation in HREC. Cells were stimulated with 10 μmol/L NECA alone (◆), or NECA plus 1 μmol/L of either a mixed 37-mer oligoribonucleotide (sham, ■) or A_{2B} ribozyme (▲). Both the amount of VEGF secreted into the medium (top) and the degree of proliferation (bottom) were decreased by the ribozyme, and not by the sham oligonucleotide control; and

FIG. 9 shows adenosine receptor antagonists reduce the degree of retinal neovascularization in the mouse pup model of oxygen-induced retinopathy. Daily IP injections of antagonists (30 mg/Kg body weight) resulted in a 54% to 70% reduction compared to untreated controls. The number of eyes examined for each condition was at least 16. *Significantly different ($p < 0.05$) from uninjected.

FIG. 10 shows the number of neovascular nuclei counted per eye section for both the uninjected and AAV-IGF1R Rz1 injected eyes.

FIG. 11 shows a schematic illustration of a representative hairpin ribozyme molecule of the present invention.

FIG. 12 shows a schematic illustration of a representative hammerhead ribozyme molecule of the present invention. The sequences of the arms may bary, as shown in Tables 4-8).

FIG. 13 shows map of AAV vector p21newHP: The hairpin ribozyme follows *Hind*III and *Spe*I cloning sites for the therapeutic hammerhead or hairpin ribozyme. This hairpin, at position 1932-2010, provides internal processing and a consistent 3' end for the active ribozyme.

FIG. 14 shows results of the injection of the IGF-1R ribozyme constructs in the oxygen-induce mouse model of ROP. Every two columns represent a minimum of 8-paired eyes. All "no injection" controls were the left eye and all injections of naked plasmids were in the right eye. The y-axis indicates the percent average number of nuclei of pre-retinal endothelial cells

per section. This number has been set to 100% for the "no injection" controls and the numbers for the plasmid injections are directly compared to their respective "no injection" control. Error bars indicate standard deviation using the student-t test and for each $P < 0.001$.

FIG. 15A and **FIG. 15B** show a summary of transfected HRECs ability to migrate in a modified Boyden chamber in response to IGF-1. FIG. 15A shows HRECs transfected with p21NewHP (diamonds), p21IGF-1R Rz1 (triangles) or p21IGF-1R Rz1i (open triangles). FIG. 15B shows HRECs transfected with p21NewHp (diamonds) or p21IGF-1R Rz2 (triangles).

FIG. 16 shows results of the injection of the A_{2B} ribozyme constructs in the oxygen-induced mouse model of ROP. Every two columns represent a minimum of 8-paired eyes. All "no injection" controls were the left eye and all injections of naked plasmids were in the right eye. The y-axis indicates the percent average number of nuclei of pre-retinal endothelial cells per section. This number has been set to 100% for the "no injection" controls and the numbers for the plasmid injections are directly compared to their respective "no injection" control. Error bars indicate standard deviation using the student-t test and for each $P < 0.001$

4. DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

Illustrative embodiments of the invention are described below. In the interest of clarity, not all features of an actual implementation are described in this specification. It will of course be appreciated that in the development of any such actual embodiment, numerous implementation-specific decisions must be made to achieve the developers' specific goals, such as compliance with system-related and business-related constraints, which will vary from one implementation to another. Moreover, it will be appreciated that such a development effort might be complex and time-consuming, but would nevertheless be a routine undertaking for those of ordinary skill in the art having the benefit of this disclosure.

In particular, the subject invention pertains in an overall and general sense to methods and compositions for achieving highly specific elimination and/or reduction of mutant and/or excess proteins or polypeptides associated with pathological conditions. Specifically exemplified herein is the use of ribozymes to treat and/or prevent diseases of the mammalian eye, and in particular, hereditary, and degenerative diseases and disorders of the human eye and its components. In one aspect, the subject invention provides catalytic RNA compositions (ribozymes) and the use of these compositions in methods for altering, decreases, reducing or eliminating the accumulation of certain proteins and polypeptides in selected cells, and in particular, those proteins and polypeptides that cause, contribute to, or exacerbate the symptoms, progression, or extent of one or more eye diseases or disorders. In particular, the

invention is focused on the species-specific cleavage of the mRNA molecules present in a cell that encode mutant proteins or polypeptides, and particularly, the specific cleavage of mRNAs that encode mutations in those proteins involved in the process of vision and normal function of the mammalian eye.

The present invention provides compositions, uses, and medicaments that exploit the catalytic properties of particular ribozyme constructs found by the inventors to be useful in the specific inactivation of mutant eye-specific or eye-localized proteins. In an overall and general sense, ribozymes are a unique class of enzymes that are comprised of ribonucleic acid (RNA). In nature, these catalytic RNA molecules conduct a variety of reactions involving RNA, including, for example, the cleavage and/or ligation of particular polynucleotide strands. The specificity of ribozymes is determined by base pairing (hydrogen bonding) between the targeting domain of the ribozyme and its complementary basepairing with a "target" sequence found within the primary sequence of the particular substrate mRNA to be cleaved. This specificity of the ribozyme for its target can be modified by altering the nucleotide sequence of the targeting domain. In fact, the catalytic domain of ribozymes (the part that actually performs the biochemical cleavage or "work"), can also be changed by altering its primary sequence, in order to either increase activity and/or the stability of the ribozyme itself.

Ribozymes, if delivered as described herein to cells of the mammalian eye, and particularly to cells of the retina and its related cellular components, by a suitable delivery means, such as for example a "gene therapy" or a gene delivery vehicle (*e.g.*, a specially designed viral vector), can provide a sustained, long-term, and even permanent treatment for a variety of retinal diseases, including, for example, retinitis pigmentosa, macular degeneration, or other pathological retina condition. Viral vectors, such as rAAV, are well known and readily available to those skilled in the art, and as such, these vectors are particularly preferred in the practice of the invention for delivery of the catalytic RNA molecules to the target mammalian cells. Utilizing the techniques of the subject invention, it is now possible to provide ribozyme compositions that can be continuously "manufactured" in the specific mammalian cells into which the rAAV vector-based constructs have been introduced (*e.g.*, retinal cells) because of the vector's ability to integrate the gene encoding the ribozyme into the animal's host DNA. Thus, catalytic therapeutic ribozymes can then be produced in the same cells that produce the mutated protein(s) causing the ocular disease. In this fashion, the cell can produce ribozymes, in effect, to correct another cellular disorder in the same cell as that which contains the ribozyme construct. This method, therefore, offers a particular advantage to long-term treatment of the

cellular dysfunction by a single, or few administrations of the viral vector constructs to the affected individual.

Ribozymes can also be used according to the subject invention as a partial treatment for recessive or semi-dominant genetic diseases of the eye as a supplement to gene replacement therapy. The delivery-expression materials and methods of the subject invention can be used to replace any gene responsible for recessive photoreceptor disease. Specific examples include the genes responsible for retinitis pigmentosa or macular degeneration. Additionally, ribozymes can be used according to the subject invention to treat RP-like disease resulting from the numerous known mutations in the rhodopsin gene. Examples of such mutations are well known to those skilled in the art. See, for example, Daiger *et al.*, *Behavioral Brain Sci.*, 18:452-67, 1995.

A further aspect of the current invention pertains to therapeutic strategies that can retard or block the effects of high glucose on progression of diabetic retinopathy. High glucose environments can result in chronically increased nitric oxide (NO) activity which leads to endothelial cell dysfunction and impaired blood retinal barrier integrity characteristic of diabetic retinopathy.

Reducing the synthesis of NOS using ribozymes can be used to retard or eliminate the damage to the blood retinal barrier. For example, ribozymes which specifically cleave mRNAs encoding VEGF, iNOS, or eNOS can be used. In specific exemplary embodiments presented herein, to inhibit the expression of iNOS and eNOS, hammerhead ribozymes that contain one long (46 nt) targeting arm 3N to the catalytic domain and a short (5 nt) targeting sequence 5N to the catalytic domain were synthesized. The "long" targeting arm of the ribozyme permits rapid association with the target sequence, while the remaining "short" arm of the ribozyme permits rapid dissociation of product necessary for catalytic turnover. It is these "arms" of the ribozyme that are selectively chosen to be complementary to one or more targeting regions on the mRNA, thereby providing a means for the ribozyme to "recognize" and thereby specifically bind to and cleave the specific mRNA for which it has been designed.

Messenger RNA molecules have a complex pattern of intramolecular hydrogen bonds that reduce the portion of the molecule available for ribozyme attack. Sites in the iNOS and eNOS mRNAs accessible to ribozyme binding can be determined using synthetic transcripts of iNOS and eNOS cDNA clones. Ribozyme cleavage can be tested on short oligonucleotides identical to sequences of accessible regions containing hammerhead target sites. The most active ribozymes can be tested on synthetic transcripts of the entire cDNA or alternatively on

total mRNA extracted from selected cells containing the targeting sequence to identify and optimize the most preferred ribozymes for treatment of a particular disorder.

As described herein, nucleic acid segments comprising one or more genes encoding the selcted ribozymes of interest are cloned into a suitable delivery vehicle (such as the rAAV vector or other viral vectors as described herein). High-potency ribozymes that cleave a specifically selected target mRNA are constructed by those skilled in the art having the benefit of the instant disclosure using any of the means known for ribozyme design and synthesis. These ribozyme constructs are then administered to particular mammalian cells, where the catalytic properties of the engineered ribozymes may be used as a therapeutic to reduce or eliminate the expression of the defective protein or polypeptide encoded by the targeted mRNA to which the specific ribozyme has been created,

For example, delivering a ribozyme specific for a mutated iNOS, eNOS, or VEGF polypeptide to selected retinal endothelial cells that produce the mutated protein, results in the reduction of expression of iNOS, eNOS, or VEGF and, ultimately, the reduction of nitric oxide production. Reduction of NO production then, in turn, reduces or delays retinal permeability, and thereby treats particular ocular dysfunction resulting from an overexpresison or accumulation of NO.

4.1 INSULIN-LIKE GROWTH FACTOR-I

IGF-I, together with platelet-derived growth factor, accounts for most of the growth-promoting activity of serum and is recognized as one of the progression factors that prompt "competence factor"-primed cells to proceed through the prereplicative phase of the cell cycle, G_1 (Clemmons, 1992). Cloning the IGF-I receptor definitively demonstrated that activation of an overexpressed IGF-I receptor could initiate mitogenesis and promote ligand-dependent neoplastic transformation. IGF-I action is tightly regulated by a series of IGF binding proteins (IGFBPs) (Guenette *et al.*, 1994; Grant and King, 1995).

While VEGF is currently viewed as the major effector for retinal neovascularization (Aiello *et al.*, 1994; Robinson *et al.*, 1996), recent studies further point to a pivotal role for IGF-I in retinal neovascularization. IGF-I receptors are present on retinal microvascular cells and these cells respond to IGF-I with a five-fold increase in DNA synthesis (King *et al.*, 1985; Grant *et al.*, 1993a). IGF-I promotes chemotaxis (migration) of human and bovine retinal endothelial cells in a concentration dependent manner (Grant *et al.*, 1987). IGF-I modulates protease expression (Grant *et al.*, 1993b) and acts as a survival factor for the retinal microvessel cells. In later stages of proliferative diabetic retinopathy, it can induce retinal angiogenesis and is

expressed by several retinal cell types in response to VEGF exposure (Punglia *et al.*, 1997). Data have demonstrated that VEGF induces IGF-I and bFGF production by HRECs.

Antibodies to IGF-I receptor, antisense strategies against IGF-I and IGF-I receptor, and dominant negative IGF-I Rc mutants all reduce cell survival and promote cell death (Beck *et al.*, 1995). Conversely, overexpression of IGF-I receptor enhances cell survival in response to death signals (Dunn *et al.*, 1997).

Altered IGF-I levels are clinically meaningful in diabetes and may be important in permitting apoptosis in response to the diabetic state. The serum level of IGF-I is reduced acutely in both clinical and experimental diabetes despite higher than normal growth hormone levels because hepatic IGF-I production requires the presence of portal insulin (Sonksen *et al.*, 1993). In streptozotocin-treated rats, there is a decrease in serum IGF-I levels and a reduction in IGF-I mRNA in liver, kidney, lung and heart during the first month of diabetes, in part due to a loss of portal insulin (Yang *et al.*, 1990). The observations that IGF-I mimics insulin's metabolic effects suggested that IGF-I could be used therapeutically to restore euglycemia. However, clinical trials with recombinant human (rh) IGF-I in patients with both insulin dependent diabetes mellitus (IDDM) and non-insulin dependent diabetes mellitus (NIDDM) were halted due to progression of retinopathy, with optic nerve neovascularization and other microvascular complications (Kolaczynski and Caro, 1994; Langford and Miell, 1993; Cusi and DeFronzo, 1995). Doses of rhIGF-I that are required to improve hyperglycemia may be limited by adverse effects and several investigators caution that rhIGF-I treatment could accelerate progression of diabetic retinopathy (Kolaczynski and Caro, 1994; Langford and Miell, 1993; Cusi and DeFronzo, 1995). Results of a recently published clinical trial in patients with severe nonproliferative diabetic retinopathy or "non high risk" proliferative diabetic retinopathy found that Octreotide, a growth hormone and IGF-I antagonist, delayed the need for laser photocoagulation.

The local tissue levels of IGF-I are probably as relevant as serum levels to the initiation of diabetic complications (Grant and King, 1995). Several clinical studies support a role for IGF-I in development of retinal neovascularization (Merimee *et al.*, 1983; Hyer *et al.*, 1989; Dills *et al.*, 1991). Studies have demonstrated a three-fold increase of IGF-I in the vitreous of diabetics with proliferative diabetic retinopathy compared to nondiabetic individuals. These findings were independently confirmed (Meyer-Schwickerath *et al.*, 1993).

This dysregulation of IGF-I may result in apoptosis as seen in nonproliferative diabetic retinopathy and proliferation as seen later in proliferative retinopathy. These studies emphasize the importance of the appropriate amount of IGF-I, since too little results in apoptosis and

acellular capillaries, too much promotes aberrant endothelial proliferation, and the appropriate amount ensures endothelial cell survival in the retina.

4.2 Adenosine and Angiogenesis

Retinal ischemia and abnormal angiogenesis occur not only in PDR, but also in retinopathy of prematurity (ROP) and in age-related macular degeneration. Substantial evidence supports a role for adenosine in promoting angiogenesis (Dusseau and Hutchins, 1988; Adair *et al.*, 1989). Studies suggest that adenosine can act as a mitogen in endothelial cells derived from various vascular beds (Sexl *et al.*, 1995; Grant *et al.*, 1999) to increase cell number, DNA synthesis (Ethier *et al.*, 1993), cell migration and vascularity (Dusseau *et al.*, 1986). Endothelial cells are known to have a very active adenosine metabolism, characterized by a large capacity for uptake and release of the nucleoside (Nees *et al.*, 1985). Adenosine can stimulate endothelial cells to alter their pattern of gene expression (Takagi *et al.*, 1996a). High levels of adenosine are associated with areas of vasculogenesis in the normal neonatal dog retina as well as sites of angiogenesis in the canine model of oxygen and induced retinopath (Taomoto *et al.*, 2000; Lutty *et al.*, 2000). Data show that the adenosine analogue NECA increases vascular endothelial cell growth factor (VEGF) mRNA in human retinal endothelial cells (HREC) (Grant *et al.*, 1999). In addition to mediating VEGF expression, adenosine has a synergistic effect with VEGF on retinal endothelial cell migration and capillary morphogenesis *in vitro* (Lutty *et al.*, 1998).

Adenosine is a critical mediator of blood flow changes in response to ischemia. It is a significant component of the retina's compensatory hyperemic response to ischemia, hypoxia, and hypoglycemia (Rego *et al.*, 1996). Increasing endogenous adenosine concentrations may be useful in ameliorating post-ischemic hypoperfusion. Current evidence suggests that adenosine is a vital component of the endogenous retinal response to substrate deprivation. Adenosine is a potent vasodilator. It has been appreciated that vasodilators increase the growth of endothelial cells while vasoconstrictors increase the growth of smooth muscle cells (Brown and Jampol, 1996). In the retinal microvasculature, adenosine and adenosine analogues cause concentration-dependent vasodilation (Gidday and Park, 1993). The vasodilatory response of retinal arterioles to hypoxia in newborn piglets is attenuated by the nonselective adenosine receptor antagonist, 8SPT. Likewise, 8SPT inhibits retinal arteriolar vasodilation induced by systemic hypotension, whereas inhibiting adenosine uptake with S(4-nitrobenzyl)-6-thioinosine (NBTI) potentiates, arteriolar dilation. Altogether, these observations strongly support a role for endogenously

released adenosine as a key mediator of blood flow during conditions of reduced O_2 supply (Gidday and Park, 1993).

In bovine retinal endothelial cells and pericytes, adenosine receptor inhibition reduces the induction of VEGF mRNA and protein expression when cells are exposed to hypoxic conditions (Takagi *et al.*, 1996b). Hypoxia-induced increases in VEGF mRNA were inhibited by adenosine deaminase, an enzyme that degrades adenosine to inosine, which does not activate adenosine receptor. The adenosine receptor antagonist, 8-phenyltheophylline (Ethier *et al.*, 1993), can block the proliferative effect of adenosine. Theophylline and 3,7-dimethyl-1-propylxanthine (DMPX), nonselective adenosine receptor antagonists, also inhibited VEGF mRNA induction following hypoxia (Hashimoto *et al.*, 1994). The weak adenosine antagonist theobromine caused significant inhibition of angiogenic activity of ovarian cancer cells and decreased VEGF production *in vitro* in these cells (Barcz *et al.*, 1998).

4.3 ADENOSINE RECEPTORS

Adenosine can interact with at least four subtypes of G-protein coupled receptors, termed A_1, A_{2A}, A_{2B} and A_3 (Shryock and Belardinelli, 1997). These receptors are encoded by distinct genes and can be differentiated based on their affinities for adenosine agonists and antagonists (Fredholm *et al.*, 1994). A_1 and A_3 adenosine receptors interact with pertussis toxin-sensitive G proteins of the G_1 and G_0 type to inhibit adenylate cyclase, whereas A_{2A} (high affinity) and A_{2B} (low affinity) adenosine receptors stimulate adenylate cyclase *via* G_s (Fredholm *et al.*, 1994). In most cell types and organ systems, adenosine activates A_1 adenosine receptors to decrease work (decrease O_2 demand), whereas A_2 adenosine receptors increase O_2 supply (Shryock and Belardinelli, 1997). Thus, adenosine, by increasing O_2 supply (activation of A_2 adenosine receptor) and by decreasing O_2 demand (activation of A_1 adenosine receptor), is an ideal candidate to rectify imbalances between O_2 supply and demand. This has led to the concept that adenosine is a protective metabolite (Shryock and Belardinelli, 1997 (FIG. 1).

Studies suggest that adenosine acting *via* the A_{2B} adenosine receptor could promote angiogenesis. Thus, A_2 adenosine receptors mediate short term increases in O_2 supply by increasing blood flow and long term by increasing vascularity (FIG. 1). Adenosine increases cAMP production and the consequences of adenylate cyclase stimulation in endothelial cells include cell shape changes, and changes in junctional permeability in addition to angiogenesis (Stelzner *et al.*, 1989; Tuder *et al.*, 1990). Signaling pathways mediating the mitogenic action of adenosine include mitogen activated protein kinase (MAPK) (Sexl *et al.*, 1997) and protein kinase A (PKA) (Takagi *et al.*, 1996b).

Investigators have identified the A_{2A} receptor as the mediator of adenosine's actions in different species. Takagi *et al.* (1996a) reported that endogenously released adenosine stimulates VEGF gene expression in bovine retinal endothelial cells and pericytes through stimulation of A_{2A} adenosine receptor. A_2 receptors are associated with vessels. Lutty demonstrated that A_{2A} receptor localized to the edge of the developing vasculature in canine retina. Taomoto and coworkers also demonstrated high levels of A_{2A} receptor immunoreactivity in immature intravitreal neovascular formations in the canine oxygen-induced retinopathy model (Taomoto *et al.*, 2000).

4.4 HYPOXIA INDUCIBLE FACTORS

Hypoxia and its subsequent tissue ischemia induce a significant increase in extracellular adenosine and hypoxanthine and to a lesser extent inosine. Local hypoglycemia, often associated with tissue hypoxia, also induces adenosine and hypoxanthine formation and release. Hypoxia also activates the expression of a number of genes, principally by the stabilization of members of the basic helical-loop-helix (bHLH)-PAS family of transcription factors that bind to the hypoxia response element (HRE), a consensus DNA sequence, the hypoxia response element (HRE). Studies of the erythropoietin (Epo) gene led to the identification of hypoxia inducible factor (HIF-1). HIF-1 is a transcription factor activated by hypoxia. HIF-1 is composed of two subunits HIF-1α and HIF-1β, both bHLH-PAS proteins. HIF-1α protein expression is rapidly induced by hypoxia and the magnitude of the response is inversely related to the cellular O_2 concentration. Dimerization with HIF-1β induces a conformational change in HIF-1α, possibly mediated by HSP90, which is required for high affinity binding to DNA. Hypoxia response elements (HREs) containing functionally essential HIF-1 binding sites were identified in genes encoding VEGF, glucose transport 1 and the glycolytic enzymes, aldolase A, enolase, lactate dehydrogenase A and phosphoglycerate kinase 1. HIF-1 has also been shown to activate transcription of genes encoding inducible nitric oxide synthase and heme oxygenase 1 which are responsible for the synthesis of the vasoactive molecules NO and CO, respectively, and transferring, which, like Epo, is essential for erythropoiesis.

The known target genes demonstrate that HIF-1 facilitates both increased O_2 delivery, by promoting erythropoiesis, angiogenesis, and vasodilation, and decreased O_2 utilization, by participating in the transition from oxidative phosphorylation to glycolysis as a means of generating ATP. The HIF-1 activation transduction pathway is poorly understood. Extracellular regulated kinases (ERK), members of the MAPK family of kinases, are activated in hypoxia. Minet and coworkers demonstrated that in human microvascular endothelial cells,

ERK kinases are activated during hypoxia. Using dominant negative mutants, they showed that ERK is needed for hypoxia induced HIF-1 transactivation activity and that HIF-1α is phosphorylated in hypoxia by an ERK-dependent pathway (Minet *et al.*, 2000). It was shown that exposure of HREC to adenosine agonists results in activation of ERK. Adenosine is released during hypoxia, thus during hypoxia adenosine may be mediating the phosphorylation of HIF by activation of ERK.

4.5 PROGENITOR ENDOTHELIAL CELLS INCORPORATE INTO SITES OF ACTIVE ANGIOGENESIS

Vasculogenesis is the *in situ* differentiation of mesodermal precursors to angioblasts that differentiate into endothelial cells to form the primitive capillary network. Vasculogenesis is limited to early embryogenesis and is believed not to occur in the adult. By contrast, angiogenesis is the sprouting of new capillaries from pre-existing blood vessels and occurs in late embryogenesis and postnatal life. The basic mechanisms underlying vasculogenesis and angiogenesis are at present unclear. Human stem cells from peripheral blood can differentiate into endothelial cells. A number of reports have demonstrated the presence of circulating endothelial cells. Asahara *et al.* showed that $CD34^+$ cells derived from peripheral circulation could form endothelial colonies (Asahara *et al.*, 1997). These were identified by their ability to incorporate acetylated LDL, express PCAM and Tie-2 receptors and produce nitric oxide following VEGF stimulation. CD34 is a marker for hematopoietic progenitor cells that give rise to all blood cells and is found on all endothelial cells in the adult and developing embryo. Thus, the hemangioblast apparently gives rise to both the hematopoietic cells and vascular cells during embryogenesis.

Putative angioblasts were isolated from the leukocyte fraction of peripheral blood and contributed to angiogenic blood vessel formation in a rabbit model of hindlimb ischemia. In these elegant studies by Asahara *et al.*, human $CD34^+$ cells were administered to C57BL/6J 129/SV background athymic nude mice. Two days after creating hindlimb ischemia by excising one femoral artery, the mice were injected with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI)-labeled human $CD34^+$ cells into their tail veins. One to six weeks later histological examination revealed numerous DiI-labeled cells in the neovascularized ischemic hindlimb (Asahara *et al.*, 1997). Nearly all labeled cells appeared integrated into capillary vessel walls. DiI-labeled cells consistently colocalized with cells immunostaining for CD31 and Tie-2, endothelial cell markers. No labeled cells were found in uninjured limbs. Similarly DiI-labeled positive for the VEGF receptor Flk^+ were administered

to C57BL/6J 129/SV background athymic nude mice after creating hindlimb ischemia, with results similar to $CD34^+$ cells. (Asahara *et al.*, 1997). These results support that circulating cells that express CD34 and Flk contribute to neoangiogenesis in adult animals, consistent with angiogenesis, a paradigm otherwise restricted to embryogenesis (Flamme and Risau, 1992).

Determining the origin of endothelial cells that form preretinal neovascularization is critical to developing nondestructive therapies to treat the condition. It is feasible to imagine that the diabetic individual may have an increased number of circulating endothelial precursor cells (EPCs) due to the abnormal hormonal and metabolic milieu associated with the diabetic state. Increased serum levels of the growth factors IGF-I and VEGF as well as of the cytokine TNF-α have been found in diabetics with proliferative diabetic retinopathy (Grant *et al.*, 1986; Limb *et al.*, 1996). These growth factors and cytokines could increase the number of circulating bone marrow (BM)-derived EPCs (Bikfalvi and Han, 1994). EPCs may home to tissue stroma in the eye for purposes of providing maintenance reservoirs of EPCs, analogous to satellite myoblasts and fibroblasts (Asahara *et al.*, 1997; Bikfalvi and Han, 1994). Local growth factors released in response to adenosine could also increase the expression of as yet unidentified cell adhesion molecules and stimulate supportive stromal cells that would likely contribute to enhanced homing of circulating EPCs to the retina. EPCs may further differentiate and/or incorporate into foci of neovascularization.

4.6 GENE THERAPY FOR DIABETIC RETINOPATHY

Recombinant adeno-associated virus (rAAV) vectors have been used successfully in long-term gene delivery to the retina (Flannery *et al.*, 1997; Bennett *et al.*, 1997), the lung (Flotte *et al.*, 1993), muscle (Kessler *et al.*, 1996; Xiao and Samulski, 1996) , brain (Klein *et al.*, 1998; Xiao *et al.*, 1997) , spinal cord (Peel *et al.*, 1997), liver (Snyder *et al.*, 1997) and blood vessels (Rolling *et al.*, 1997). In particular, AAV infects vascular endothelial cells *in vivo* (Gnatenko *et al.*, 1997; Lynch *et al.*, 1997). A single intravitreal injection of AAV expressing marker proteins (β-galactosidase or *gfp*) was able to genetically transduce a variety of cell types in the guinea pig eye, including blood vessels, for up to one year post injection with no evidence of inflammation or other abnormalities (Guy *et al.*, 1999). Unlike adenovirus vectors used in gene therapy trials, AAV does not cause inflammation and does not provoke a cell mediated immune response (Bennett *et al.*, 1997; Bennett *et al.*, 1999). Unlike retroviral vectors, AAV is able to infect non-cycling cells, such as vascular endothelial cells. Retroviral vectors have been used for dividing endothelial cells in culture, but not for non-dividing cells *in vivo*.

A low therapeutic index (ratio of toxic dose to therapeutic dose) is important for gene-based therapies, and one approach to achieve this has been transcriptional targeting through use of tissue-specific regulatory elements. For example, we have employed the rhodopsin promoter to achieve photoreceptor-specific expression of ribozymes in rats (Lewin *et al.*, 1998). A more versatile approach might be to use a regulatory element that is controlled by a condition common to a broad range of diseases, *i.e.*, ischemia. Ischemia is characteristic of a number of pathologies ranging from vascular occlusion to cancer. Consequently, several research groups are developing vectors for gene delivery that employ regulation by the hypoxia response element (HRE). These cis-acting elements have been identified as enhancer elements in the 5' or 3' flanking region of a variety of hypoxia-regulated genes, but have been best characterized in the context of the genes for erythropoietin, VEGF and the glycolytic enzyme phosphoglycerate kinase 1 (PGK1). A hypoxia-regulated element from the PGK1 gene showed a 50-fold induction upstream of a minimal SV40 promoter in the context of either an adenoviral vector (Binley *et al.*, 1999) or a retroviral vector (Boast *et al.*, 1999). These levels of expression of marker genes (β-galactosidase and luciferase, respectively) were equivalent to the unregulated expression of the same genes in the same cultured cells directed by the cytomegalovirus (CMV) immediate early promoter. Hypoxia-regulated vectors may have utility for restricting the delivery of therapeutic proteins to ischemic sites. The fact that progenitor endothelial cells home to sites of ischemia suggests potential utility as autologous vectors for gene therapy. For antiangiogenic therapies, $CD34^+$ cells could be transfected with angiogenesis inhibitors.

4.7 RIBOZYMES

Although proteins traditionally have been used for catalysis of nucleic acids, another class of macromolecules has emerged as useful in this endeavor. Ribozymes are RNA-protein complexes that cleave nucleic acids in a site-specific fashion. Ribozymes have specific catalytic domains that possess endonuclease activity (Kim and Cech, 1987; Gerlach *et al.*, 1987; Forster and Symons, 1987). For example, a large number of ribozymes accelerate phosphoester transfer reactions with a high degree of specificity, often cleaving only one of several phosphoesters in an oligonucleotide substrate (Cech *et al.*, 1981; Michel and Westhof, 1990; Reinhold-Hurek and Shub, 1992). This specificity has been attributed to the requirement that the substrate bind *via* specific base-pairing interactions to the internal guide sequence ("IGS") of the ribozyme prior to chemical reaction.

Ribozyme catalysis has primarily been observed as part of sequence-specific cleavage/ligation reactions involving nucleic acids (Joyce, 1989; Cech *et al.*, 1981). For

example, U. S. Patent No. 5,354,855 (specifically incorporated herein by reference) reports that certain ribozymes can act as endonucleases with a sequence specificity greater than that of known ribonucleases and approaching that of the DNA restriction enzymes. Thus, sequence-specific ribozyme-mediated inhibition of gene expression may be particularly suited to therapeutic applications (Scanlon *et al.*, 1991; Sarver *et al.*, 1990). Recently, it was reported that ribozymes elicited genetic changes in some cells lines to which they were applied; the altered genes included the oncogenes H-*ras*, c-*fos* and genes of HIV. Most of this work involved the modification of a target mRNA, based on a specific mutant codon that is cleaved by a specific ribozyme.

Six basic varieties of naturally occurring enzymatic RNAs are known presently. Each can catalyze the hydrolysis of RNA phosphodiester bonds *in trans* (and thus can cleave other RNA molecules) under physiological conditions. In general, enzymatic nucleic acids act by first binding to a target RNA. Such binding occurs through the target binding portion of a enzymatic nucleic acid which is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the enzymatic nucleic acid first recognizes and then binds a target RNA through complementary base pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved its RNA target, it is released from that RNA to search for another target and can repeatedly bind and cleave new targets.

The enzymatic nature of a ribozyme is advantageous over many technologies, such as antisense technology (where a nucleic acid molecule simply binds to a nucleic acid target to block its translation) since the concentration of ribozyme necessary to affect a therapeutic treatment is lower than that of an antisense oligonucleotide. This advantage reflects the ability of the ribozyme to act enzymatically. Thus, a single ribozyme molecule is able to cleave many molecules of target RNA. In addition, the ribozyme is a highly specific inhibitor, with the specificity of inhibition depending not only on the base pairing mechanism of binding to the target RNA, but also on the mechanism of target RNA cleavage. Single mismatches, or base-substitutions, near the site of cleavage can completely eliminate catalytic activity of a ribozyme. Similar mismatches in antisense molecules do not prevent their action (Woolf *et al.*, 1992). Thus, the specificity of action of a ribozyme is greater than that of an antisense oligonucleotide binding the same RNA site.

The enzymatic nucleic acid molecule may be formed in a hammerhead, hairpin, a hepatitis δ virus, group I intron or RNaseP RNA (in association with an RNA guide sequence)

or Neurospora VS RNA motif. Examples of hammerhead motifs are described by Rossi *et al.* (1992). Examples of hairpin motifs are described by Hampel *et al.* (Eur. Pat. Appl. Publ. No. EP 0360257), Hampel and Tritz (1989), Hampel *et al.* (1990) and U. S. Patent 5,631,359 (specifically incorporated herein by reference). An example of the hepatitis δ virus motif is described by Perrotta and Been (1992); an example of the RNaseP motif is described by Guerrier-Takada *et al.* (1983); Neurospora VS RNA ribozyme motif is described by Collins (Saville and Collins, 1990; Saville and Collins, 1991; Collins and Olive, 1993); and an example of the Group I intron is described in U. S. Patent 4,987,071 (specifically incorporated herein by reference). All that is important in an enzymatic nucleic acid molecule of this invention is that it has a specific substrate binding site which is complementary to one or more of the target gene RNA regions, and that it have nucleotide sequences within or surrounding that substrate binding site which impart an RNA cleaving activity to the molecule. Thus the ribozyme constructs need not be limited to specific motifs mentioned herein.

In certain embodiments, it may be important to produce enzymatic cleaving agents that exhibit a high degree of specificity for the RNA of a desired target, such as one of the sequences disclosed herein. The enzymatic nucleic acid molecule is preferably targeted to a highly conserved sequence region of a target mRNA. Such enzymatic nucleic acid molecules can be delivered exogenously to specific cells as required, although in preferred embodiments the ribozymes are expressed from DNA or RNA vectors that are delivered to specific cells.

Small enzymatic nucleic acid motifs (*e.g.*, of the hammerhead or the hairpin structure) may also be used for exogenous delivery. The simple structure of these molecules increases the ability of the enzymatic nucleic acid to invade targeted regions of the mRNA structure. Alternatively, catalytic RNA molecules can be expressed within cells from eukaryotic promoters (*e.g.*, Scanlon *et al.*, 1991; Kashani-Sabet *et al.*, 1992; Dropulic *et al.*, 1992; Weerasinghe *et al.*, 1991; Ojwang *et al.*, 1992; Chen *et al.*, 1992; Sarver *et al.*, 1990). Those skilled in the art realize that any ribozyme can be expressed in eukaryotic cells from the appropriate DNA vector. The activity of such ribozymes can be augmented by their release from the primary transcript by a second ribozyme (Int. Pat. Appl. Publ. No. WO 93/23569, and Int. Pat. Appl. Publ. No. WO 94/02595, both hereby incorporated by reference; Ohkawa *et al.*, 1992; Taira *et al.*, 1991; and Ventura *et al.*, 1993).

Ribozymes may be added directly, or can be complexed with cationic lipids, lipid complexes, packaged within liposomes, or otherwise delivered to target cells. The RNA or RNA complexes can be locally administered to relevant tissues *ex vivo*, or *in vivo* through

injection, aerosol inhalation, infusion pump or stent, with or without their incorporation in biopolymers.

Ribozymes may be designed as described in Int. Pat. Appl. Publ. No. WO 93/23569 and Int. Pat. Appl. Publ. No. WO 94/02595 (each specifically incorporated herein by reference) and synthesized to be tested *in vitro* and *in vivo*, as described. Such ribozymes can also be optimized for delivery. While specific examples are provided, those in the art will recognize that equivalent RNA targets in other species can be utilized when necessary.

Hammerhead or hairpin ribozymes may be individually analyzed by computer folding (Jaeger *et al.*, 1989) to assess whether the ribozyme sequences fold into the appropriate secondary structure, as described herein. Those ribozymes with unfavorable intramolecular interactions between the binding arms and the catalytic core are eliminated from consideration. Varying binding arm lengths can be chosen to optimize activity. Generally, at least 5 or so bases on each arm are able to bind to, or otherwise interact with, the target RNA.

Ribozymes of the hammerhead or hairpin motif may be designed to anneal to various sites in the mRNA message, and can be chemically synthesized. The method of synthesis used follows the procedure for normal RNA synthesis as described in Usman *et al.* (1987) and in Scaringe *et al.* (1990) and makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5′-end, and phosphoramidites at the 3′-end. Average stepwise coupling yields are typically >98%. Hairpin ribozymes may be synthesized in two parts and annealed to reconstruct an active ribozyme (Chowrira and Burke, 1992). Ribozymes may be modified extensively to enhance stability by modification with nuclease resistant groups, for example, 2′-amino, 2′-C-allyl, 2′-flouro, 2′-o-methyl, 2′-H (for a review see *e.g.*, Usman and Cedergren, 1992). Ribozymes may be purified by gel electrophoresis using general methods or by high-pressure liquid chromatography and resuspended in water.

Ribozyme activity can be optimized by altering the length of the ribozyme binding arms, or chemically synthesizing ribozymes with modifications that prevent their degradation by serum ribonucleases (see *e.g.*, Int. Pat. Appl. Publ. No. WO 92/07065; Perrault *et al.*, 1990; Pieken *et al.*, 1991; Usman and Cedergren, 1992; Int. Pat. Appl. Publ. No. WO 93/15187; Int. Pat. Appl. Publ. No. WO 91/03162; Eur. Pat. Appl. Publ. No. 92110298.4; U.S. Patent 5,334,711; and Int. Pat. Appl. Publ. No. WO 94/13688, which describe various chemical modifications that can be made to the sugar moieties of enzymatic RNA molecules), modifications which enhance their efficacy in cells, and removal of stem II bases to shorten RNA synthesis times and reduce chemical requirements.

A preferred means of accumulating high concentrations of a ribozyme(s) within cells is to incorporate the ribozyme-encoding sequences into a DNA expression vector. Transcription of the ribozyme sequences are driven from a promoter for eukaryotic RNA polymerase I (pol I), RNA polymerase II (pol II), or RNA polymerase III (pol III). Transcripts from pol II or pol III promoters will be expressed at high levels in all cells; the levels of a given pol II promoter in a given cell type will depend on the nature of the gene regulatory sequences (enhancers, silencers, *etc.*) present nearby. Prokaryotic RNA polymerase promoters may also be used, providing that the prokaryotic RNA polymerase enzyme is expressed in the appropriate cells (Elroy-Stein and Moss, 1990; Gao and Huang, 1993; Lieber *et al.*, 1993; Zhou *et al.*, 1990). Ribozymes expressed from such promoters can function in mammalian cells (Kashani-Sabet *et al.*, 1992; Ojwang *et al.*, 1992; Chen *et al.*, 1992; Yu *et al.*, 1993; L'Huillier *et al.*, 1992; Lisziewicz *et al.*, 1993). Although incorporation of the present ribozyme constructs into adeno-associated viral vectors is preferred, such transcription units can be incorporated into a variety of vectors for introduction into mammalian cells, including but not restricted to, plasmid DNA vectors, other viral DNA vectors (such as adenovirus vectors), or viral RNA vectors (such as retroviral, semliki forest virus, sindbis virus vectors).

Sullivan *et al.* (Int. Pat. Appl. Publ. No. WO 94/02595) describes general methods for delivery of enzymatic RNA molecules. Ribozymes may be administered to cells by a variety of methods known to those familiar to the art, including, but not restricted to, encapsulation in liposomes, by iontophoresis, or by incorporation into other vehicles, such as hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres. For some indications, ribozymes may be directly delivered *ex vivo* to cells or tissues with or without the aforementioned vehicles. Alternatively, the RNA/vehicle combination may be locally delivered by direct inhalation, by direct injection or by use of a catheter, infusion pump or stent. Other routes of delivery include, but are not limited to, intravascular, intramuscular, subcutaneous or joint injection, aerosol inhalation, oral (tablet or pill form), topical, systemic, ocular, intraocular, retinal, subretinal, intraperitoneal and/or intrathecal delivery. More detailed descriptions of ribozyme and rAAV vector delivery and administration are provided in Int. Pat. Appl. Publ. No. WO 94/02595 and Int. Pat. Appl. Publ. No. WO 93/23569, each specifically incorporated herein by reference.

Ribozymes and the AAV vectored-constructs of the present invention may be used to inhibit gene expression and define the role (essentially) of specified gene products in the progression of one or more retinal diseases and/or disorders. In this manner, other genetic targets may be defined as important mediators of the disease. These studies lead to better

treatment of the disease progression by affording the possibility of combination therapies (*e.g.*, multiple ribozymes targeted to different genes, ribozymes coupled with known small molecule inhibitors, or intermittent treatment with combinations of ribozymes and/or other chemical or biological molecules).

4.8 PROMOTERS AND ENHANCERS

Recombinant vectors form important aspects of the present invention. The term "expression vector or construct" means any type of genetic construct containing a nucleic acid in which part or all of the nucleic acid encoding sequence is capable of being transcribed. In preferred embodiments, expression only includes transcription of the nucleic acid, for example, to generate ribozyme constructs.

Particularly useful vectors are contemplated to be those vectors in which the nucleic acid segment to be transcribed is positioned under the transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrases "operatively positioned," "under the control" or "under the transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene.

In preferred embodiments, it is contemplated that certain advantages will be gained by positioning the coding DNA segment under the control of a recombinant, or heterologous, promoter. As used herein, a recombinant or heterologous promoter is intended to refer to a promoter that is not normally associated with a ribozyme construct in its natural environment. Such promoters may include promoters normally associated with other genes, and/or promoters isolated from any other bacterial, viral, eukaryotic, or mammalian cell.

Naturally, it will be important to employ a promoter that effectively directs the expression of the DNA segment in the cell type, organism, or even animal, chosen for expression. The use of promoter and cell type combinations for protein expression is generally known to those of skill in the art of molecular biology; for example, see Sambrook *et al.* (1989), incorporated herein by reference. The promoters employed may be constitutive, or inducible, and can be used under the appropriate conditions to direct high-level expression of the introduced DNA segment.

At least one module in a promoter functions to position the start site for RNA synthesis. The best-known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase

gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

The particular promoter that is employed to control the expression of a nucleic acid is not believed to be critical, so long as it is capable of expressing the nucleic acid in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the nucleic acid coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter, such as a CMV or an HSV promoter. In certain aspects of the invention, tetracycline controlled promoters are contemplated.

In various other embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter and the Rous sarcoma virus long terminal repeat can be used to obtain high-level expression of transgenes. The use of other viral or mammalian cellular or bacterial phage promoters that are well known in the art to achieve expression of a transgene is contemplated as well, provided that the levels of expression are sufficient for a given purpose. Tables 1 and 2 below list several elements/promoters that may be employed, in the context of the present invention, to regulate the expression of the present ribozyme constructs. This list is not intended to be exhaustive of all the possible elements involved in the promotion of transgene expression but, merely, to be exemplary thereof.

Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization.

Additionally any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression. Use of a T3, T7 or SP6 cytoplasmic expression system is another possible embodiment. Eukaryotic cells can support cytoplasmic transcription from certain bacterial promoters if the appropriate bacterial polymerase is provided, either as part of the delivery complex or as an additional genetic expression construct.

TABLE 1

PROMOTER AND ENHANCER ELEMENTS

PROMOTER/ENHANCER	REFERENCES
Immunoglobulin Heavy Chain	Banerji *et al.*, 1983; Gilles *et al.*, 1983; Grosschedl and Baltimore, 1985; Atchinson and Perry, 1986, 1987; Imler *et al.*, 1987; Weinberger *et al.*, 1984; Kiledjian *et al.*, 1988; Porton *et al.*; 1990
Immunoglobulin Light Chain	Queen and Baltimore, 1983; Picard and Schaffner, 1984
T-Cell Receptor	Luria *et al.*, 1987; Winoto and Baltimore, 1989; Redondo *et al.*, 1990
HLA DQ a and DQ β	Sullivan and Peterlin, 1987
β-Interferon	Goodbourn *et al.*, 1986; Fujita *et al.*, 1987; Goodbourn and Maniatis, 1988
Interleukin-2	Greene *et al.*, 1989
Interleukin-2 Receptor	Greene *et al.*, 1989; Lin *et al.*, 1990
MHC Class II 5	Koch *et al.*, 1989
MHC Class II HLA-DRa	Sherman *et al.*, 1989
β-Actin	Kawamoto *et al.*, 1988; Ng *et al.*; 1989
Muscle Creatine Kinase	Jaynes *et al.*, 1988; Horlick and Benfield, 1989; Johnson *et al.*, 1989
Prealbumin (Transthyretin)	Costa *et al.*, 1988
Elastase I	Ornitz *et al.*, 1987
Metallothionein	Karin *et al.*, 1987; Culotta and Hamer, 1989
Collagenase	Pinkert *et al.*, 1987; Angel *et al.*, 1987
Albumin Gene	Pinkert *et al.*, 1987; Tronche *et al.*, 1989, 1990

TABLE 1 (con't)

PROMOTER/ENHANCER	REFERENCES
α-Fetoprotein	Godbout *et al.*, 1988; Campere and Tilghman, 1989
t-Globin	Bodine and Ley, 1987; Perez-Stable and Constantini, 1990
β-Globin	Trudel and Constantini, 1987
e-fos	Cohen *et al.*, 1987
c-HA-ras	Treisman, 1986; Deschamps *et al.*, 1985
Insulin	Edlund *et al.*, 1985
Neural Cell Adhesion Molecule (NCAM)	Hirsh *et al.*, 1990
α_1-Antitrypain	Latimer *et al.*, 1990
H2B (TH2B) Histone	Hwang *et al.*, 1990
Mouse or Type I Collagen	Ripe *et al.*, 1989
Glucose-Regulated Proteins (GRP94 and GRP78)	Chang *et al.*, 1989
Rat Growth Hormone	Larsen *et al.*, 1986
Human Serum Amyloid A (SAA)	Edbrooke *et al.*, 1989
Troponin I (TN I)	Yutzey *et al.*, 1989
Platelet-Derived Growth Factor	Pech *et al.*, 1989
Duchenne Muscular Dystrophy	Klamut *et al.*, 1990
SV40	Banerji *et al.*, 1981; Moreau *et al.*, 1981; Sleigh and Lockett, 1985; Firak and Subramanian, 1986; Herr and Clarke, 1986; Imbra and Karin, 1986; Kadesch and Berg, 1986; Wang and Calame, 1986; Ondek *et al.*, 1987; Kuhl *et al.*, 1987; Schaffner *et al.*, 1988
Polyoma	Swartzendruber and Lehman, 1975; Vasseur *et al.*, 1980; Katinka *et al.*, 1980, 1981; Tyndall *et al.*, 1981; Dandolo *et al.*, 1983; de Villiers *et al.*, 1984; Hen *et al.*, 1986; Satake *et al.*, 1988; Campbell and Villarreal, 1988
Retroviruses	Kriegler and Botchan, 1982, 1983; Levinson *et al.*, 1982; Kriegler *et al.*, 1983, 1984a, b, 1988; Bosze *et al.*, 1986; Miksicek *et al.*, 1986; Celander and Haseltine, 1987; Thiesen *et al.*, 1988; Celander *et al.*, 1988; Choi *et al.*, 1988; Reisman and Rotter, 1989
Papilloma Virus	Campo *et al.*, 1983; Lusky *et al.*, 1983; Spandidos and Wilkie, 1983; Spalholz *et al.*, 1985; Lusky and Botchan, 1986; Cripe *et al.*, 1987; Gloss *et al.*, 1987; Hirochika *et al.*, 1987; Stephens and Hentschel, 1987; Glue *et al.*, 1988
Hepatitis B Virus	Bulla and Siddiqui, 1986; Jameel and Siddiqui, 1986; Shaul and Ben-Levy, 1987; Spandau and Lee, 1988; Vannice and Levinson, 1988

TABLE 1 (con't)

PROMOTER/ENHANCER	REFERENCES
Human Immunodeficiency Virus	Muesing *et al.*, 1987; Hauber and Cullan, 1988; Jakobovits *et al.*, 1988; Feng and Holland, 1988; Takebe *et al.*, 1988; Rosen *et al.*, 1988; Berkhout *et al.*, 1989; Laspia *et al.*, 1989; Sharp and Marciniak, 1989; Braddock *et al.*, 1989
Cytomegalovirus	Weber *et al.*, 1984; Boshart *et al.*, 1985; Foecking and Hofstetter, 1986
Gibbon Ape Leukemia Virus	Holbrook *et al.*, 1987; Quinn *et al.*, 1989

TABLE 2

INDUCIBLE ELEMENTS

ELEMENT	INDUCER	REFERENCES
MT II	Phorbol Ester (TFA) Heavy metals	Palmiter *et al.*, 1982; Haslinger and Karin, 1985; Searle *et al.*, 1985; Stuart *et al.*, 1985; Imagawa *et al.*, 1987, Karin *et al.*, 1987; Angel *et al.*, 1987b; McNeall *et al.*, 1989
MMTV (mouse mammary tumor virus)	Glucocorticoids	Huang *et al.*, 1981; Lee *et al.*, 1981; Majors and Varmus, 1983; Chandler *et al.*, 1983; Lee *et al.*, 1984; Ponta *et al.*, 1985; Sakai *et al.*, 1988
β-Interferon	poly(rI)x poly(rc)	Tavernier *et al.*, 1983
Adenovirus 5 <u>E2</u>	Ela	Imperiale and Nevins, 1984
Collagenase	Phorbol Ester (TPA)	Angel *et al.*, 1987a
Stromelysin	Phorbol Ester (TPA)	Angel *et al.*, 1987b
SV40	Phorbol Ester (TPA)	Angel *et al.*, 1987b
Murine MX Gene	Interferon, Newcastle Disease Virus	
GRP78 Gene	A23187	Resendez *et al.*, 1988
α-2-Macroglobulin	IL-6	Kunz *et al.*, 1989
Vimentin	Serum	Rittling *et al.*, 1989
MHC Class I Gene H-2κb	Interferon	Blanar *et al.*, 1989
HSP70	Ela, SV40 Large T Antigen	Taylor *et al.*, 1989; Taylor and Kingston, 1990a,b
Proliferin	Phorbol Ester-TPA	Mordacq and Linzer, 1989
Tumor Necrosis Factor	FMA	Hensel *et al.*, 1989
Thyroid Stimulating Hormone a Gene	Thyroid Hormone	Chatterjee *et al.*, 1989

As used herein, the terms "engineered" and "recombinant" cells are intended to refer to a cell into which an exogenous DNA segment, such as DNA segment that leads to the

transcription of a ribozyme, has been introduced. Therefore, engineered cells are distinguishable from naturally occurring cells, which do not contain a recombinantly introduced exogenous DNA segment. Engineered cells are thus cells having DNA segment introduced through the hand of man.

To express a ribozyme in accordance with the present invention one would prepare an expression vector that comprises a ribozyme-encoding nucleic acid under the control of one or more promoters. To bring a sequence "under the control of" a promoter, one positions the 5' end of the transcription initiation site of the transcriptional reading frame generally between about 1 and about 50 nucleotides "downstream" of (*i.e.*, 3' of) the chosen promoter. The "upstream" (*i.e.*, 5') promoter stimulates transcription of the DNA and promotes expression of the encoded ribozyme. This is an exemplary meaning of "recombinant expression" when used in the context of the present invention.

4.9 ADENO-ASSOCIATED VIRUS (AAV)

Adeno-associated virus (AAV) is particularly attractive for gene transfer because it does not induce any pathogenic response and can integrate into the host cellular chromosome (Kotin *et al.*, 1990). The AAV terminal repeats (TRs) are the only essential *cis*-components for the chromosomal integration (Muzyczka and McLaughin, 1988). These TRs are reported to have promoter activity (Flotte *et al.*, 1993). They may promote efficient gene transfer from the cytoplasm to the nucleus or increase the stability of plasmid DNA and enable longer-lasting gene expression (Bartlett *et al.*, 1996). Studies using recombinant plasmid DNAs containing AAV TRs have attracted considerable interest. AAV-based plasmids have been shown to drive higher and longer transgene expression than the identical plasmids lacking the TRs of AAV in most cell types (Philip *et al.*, 1994; Shafron *et al.*, 1998; Wang *et al.*, 1999).

AAV (Ridgeway, 1988; Hermonat and Muzyczka, 1984) is a parovirus, discovered as a contamination of adenoviral stocks. It is a ubiquitous virus (antibodies are present in 85% of the US human population) that has not been linked to any disease. It is also classified as a dependovirus, because its replication is dependent on the presence of a helper virus, such as adenovirus. Five serotypes have been isolated, of which AAV-2 is the best characterized. AAV has a single-stranded linear DNA that is encapsidated into capsid proteins VP1, VP2 and VP3 to form an icosahedral virion of 20 to 24 nm in diameter (Muzyczka and McLaughlin, 1988).

The AAV DNA is approximately 4.7 kilobases long. It contains two open reading frames and is flanked by two ITRs. There are two major genes in the AAV genome: *rep* and *cap*. The *rep* gene encodes a protein responsible for viral replications, whereas the *cap* gene

encodes the capsid protein VP1-3. Each ITR forms a T-shaped hairpin structure. These terminal repeats are the only essential *cis* components of the AAV for chromosomal integration. Therefore, the AAV can be used as a vector with all viral coding sequences removed and replaced by the cassette of genes for delivery. Three viral promoters have been identified and named p5, p19, and p40, according to their map position. Transcription from p5 and p19 results in production of rep proteins, and transcription from p40 produces the capsid proteins (Hermonat and Muzyczka, 1984).

There are several factors that prompted researchers to study the possibility of using rAAV as an expression vector. One is that the requirements for delivering a gene to integrate into the host chromosome are surprisingly few. It is necessary to have the 145-bp ITRs, which are only 6% of the AAV genome. This leaves room in the vector to assemble a 4.5-kb DNA insertion. While this carrying capacity may prevent the AAV from delivering large genes, it is amply suited for delivering the antisense constructs of the present invention.

AAV is also a good choice of delivery vehicles due to its safety. There is a relatively complicated rescue mechanism: not only wild type adenovirus but also AAV genes are required to mobilize rAAV. Likewise, AAV is not pathogenic and not associated with any disease. The removal of viral coding sequences minimizes immune reactions to viral gene expression, and therefore, rAAV does not evoke an inflammatory response. AAV therefore, represents an ideal candidate for delivery of the present hammerhead ribozyme constructs.

4.10 PHARMACEUTICAL COMPOSITIONS AND KITS

Pharmaceutical compositions of the present invention will generally comprise an effective amount of at least a first ribozyme, a pair of ribozymes, or a plurality of ribozymes, incorporated into at least a first adeno-associated viral vector, or adeno-associated viral particles containing at least a first ribozyme, a pair of ribozymes, or a plurality of ribozymes, dissolved or dispersed in one or more pharmaceutically acceptable carriers, buffers, solutions, vehicles, or aqueous media.

The phrases "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, or a human, as appropriate. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic

compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

4.10.1 PARENTERAL FORMULATIONS

The ribozymes, compositions, virus, and AAV-based vectors of the present invention will often be formulated for parenteral administration, *e.g.*, formulated for injection *via* the intravenous, intramuscular, sub-cutaneous or other such routes. The preparation of an aqueous composition that contains one or more agents, such as a ribozyme, a plurality of ribozymes, a AAV vector, or one or more or adeno-associated virus particles containing one or more such ribozymes, will be known to those of skill in the art in light of the present disclosure. Typically, such compositions can be prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for using to prepare solutions or suspensions upon the addition of a liquid prior to injection can also be prepared; and the preparations can also be emulsified.

Solutions of the active compounds as freebase or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

Compositions comprising the agents of the present invention can be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts include the acid addition salts and those formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), dimethylsulfoxide (DMSO), suitable mixtures thereof, and vegetable oils. The proper

fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying techniques, which yield a powder of the active ingredient, plus any additional desired ingredient from a previously sterile-filtered solution thereof. Such injectable solutions may be used, for example, in one or more of the well known surgical methods for directly injecting compounds into the eye, or the subretinal space.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is biologically or therapeutically effective. Formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but drug release capsules and the like can also be employed.

Suitable pharmaceutical compositions in accordance with the invention will generally include an amount of one or more of the agents of the present invention admixed with an acceptable pharmaceutical diluent or excipient, such as a sterile aqueous solution, to give a range of final concentrations, depending on the intended use. The techniques of preparation is generally well known in the art as exemplified by Remington's Pharmaceutical Sciences, 16th Ed. Mack Publishing Company, 1980, incorporated herein by reference. It should be appreciated that endotoxin contamination should be kept minimally at a safe level, for example, less that 0.5 ng/mg protein. Moreover, for human administration, preparations should meet sterility, pyrogenicity, and general safety and purity standards as required by FDA Office of Biological Standards.

In addition to the compounds formulated for parenteral administration, such as intravenous or intramuscular injection, other pharmaceutically acceptable forms are also

contemplated, *e.g.*, time release or sustained-release formulations, liposomal formulations, microspheres, nanocapsules, and the like. Other pharmaceutical formulations may also be used, dependent on the condition to be treated. Of course, methods for the determination of optimal dosages for conditions such as these would be evident to those of skill in the art in light of the instant specification, and the knowledge of the skilled artisan.

It is contemplated that certain benefits will result from the manipulation of the agents of the present invention to provide them with a longer *in vivo* half-life. Slow release formulations are generally designed to give a constant drug level over an extended period. Increasing the half-life of a drug, such as agents of the present invention, is intended to result in high intracellular levels upon administration, which levels are maintained for a longer time, but which levels generally decay depending on the pharmacokinetics of the construct.

4.10.2 THERAPEUTIC KITS

The present invention also provides therapeutic kits comprising the agents of the present invention described herein. Such kits will generally contain, in suitable container, a pharmaceutically acceptable formulation of at least a first ribozyme, plurality of ribozymes or adeno-associated virus particles comprising at least a first ribozyme or a plurality of ribozymes, in accordance with the invention. The kits may also contain other pharmaceutically acceptable formulations.

The kits may have a single container that contains the agent, with or without any additional components, or they may have distinct container means for each desired agent. In such kits, the components may be pre-complexed, either in a molar equivalent combination, or with one component in excess of the other; or each of the components of the kit may be maintained separately within distinct containers prior to administration to a patient.

When the components of the kit are provided in one or more liquid solutions, the liquid solution is an aqueous solution, with a sterile aqueous solution being particularly preferred. However, the components of the kit may be provided as dried powder(s). When reagents or components are provided as a dry powder, the powder can be reconstituted by the addition of a suitable solvent. It is envisioned that the solvent may also be provided in another container means. One of the components of the kit may be provided in sealed vials, syringes, or ampules for direct ocular administration.

The container means of the kit will generally include at least one vial, test tube, flask, bottle, syringe or other container means, into which a ribozyme, a plurality of ribozymes, or an AAV vector compositions, or one or more adeno-associated viral particles comprising a

ribozyme or plurality of ribozymes, and any other desired agent, may be placed and, preferably, suitably aliquoted. Where additional components are included, the kit will also generally contain a second vial or other container into which these are placed, enabling the administration of separated designed doses. The kits may also comprise a second/third container means for containing a sterile, pharmaceutically acceptable buffer or other diluent.

The kits may also contain a means by which to administer the ribozyme, plurality of ribozymes, AAV-vectors ribozyme, or one or more adeno-associated viral particles comprising one or more of such ribozymes to an animal or patient, *e.g.*, one or more needles or syringes, or even an eye dropper, pipette, or other such like apparatus, from which the formulation may be injected into the animal or applied to a diseased area of the body. The kits of the present invention will also typically include a means for containing the vials, or such like, and other component, in close confinement for commercial sale, such as, *e.g.*, injection or blow-molded plastic containers into which the desired vials and other apparatus are placed and retained.

4.11 MUTAGENESIS AND PREPARATION OF MODIFIED RIBOZYME COMPOSITIONS

Site-specific mutagenesis is a technique useful in the preparation and testing of sequence variants by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

In general, the technique of site-specific mutagenesis is well known in the art. As will be appreciated, the technique typically employs a bacteriophage vector that exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage vectors are commercially available and their use is generally well known to those skilled in the art. Double stranded plasmids are also routinely employed in site directed mutagenesis, which eliminates the step of transferring the gene of interest from a phage to a plasmid.

In general, site-directed mutagenesis is performed by first obtaining a single-stranded vector, or melting of two strands of a double stranded vector that includes within its sequence a DNA sequence encoding the desired ribozyme or other nucleic acid construct. An oligonucleotide primer bearing the desired mutated sequence is synthetically prepared. This

primer is then annealed with the single-stranded DNA preparation, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli* cells, and clones are selected that include recombinant vectors bearing the mutated sequence arrangement.

The preparation of sequence variants of the selected ribozyme using site-directed mutagenesis is provided as a means of producing potentially useful species and is not meant to be limiting, as there are other ways in which sequence variants may be obtained. For example, recombinant vectors encoding the desired gene may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants.

4.12 Nucleic Acid Amplification

Nucleic acid, used as a template for amplification, may be isolated from cells contained in the biological sample according to standard methodologies (Sambrook *et al.*, 1989). The nucleic acid may be genomic DNA or fractionated or whole cell RNA. Where RNA is used, it may be desired to convert the RNA to a complementary DNA. In one embodiment, the RNA is whole cell RNA and is used directly as the template for amplification.

Pairs of primers that selectively hybridize to nucleic acids corresponding to the ribozymes or conserved flanking regions are contacted with the isolated nucleic acid under conditions that permit selective hybridization. The term "primer", as defined herein, is meant to encompass any nucleic acid that is capable of priming the synthesis of a nascent nucleic acid in a template-dependent process. Typically, primers are oligonucleotides from ten to twenty base pairs in length, but longer sequences can be employed. Primers may be provided in double-stranded or single-stranded form, although the single-stranded form is preferred.

Once hybridized, the nucleic acid:primer complex is contacted with one or more enzymes that facilitate template-dependent nucleic acid synthesis. Multiple rounds of amplification, also referred to as "cycles," are conducted until a sufficient amount of amplification product is produced.

Next, the amplification product is detected. In certain applications, the detection may be performed by visual means. Alternatively, the detection may involve indirect identification of the product via chemiluminescence, radioactive scintigraphy of incorporated radiolabel or fluorescent label or even *via* a system using electrical or thermal impulse signals (*e.g.*, Affymax technology).

A number of template dependent processes are available to amplify the marker sequences present in a given template sample. One of the best-known amplification methods is the polymerase chain reaction (referred to as PCR™), which is described in detail in U. S. Patent No. 4,683,195, U. S. Patent No. 4,683,202 and U. S. Patent No. 4,800,159 (each of which is incorporated herein by reference in its entirety).

Briefly, in PCR™, two primer sequences are prepared that are complementary to regions on opposite complementary strands of the marker sequence. An excess of deoxynucleoside triphosphates is added to a reaction mixture along with a DNA polymerase, *e.g.*, *Taq* polymerase. If the marker sequence is present in a sample, the primers will bind to the marker and the polymerase will cause the primers to be extended along the marker sequence by adding on nucleotides. By raising and lowering the temperature of the reaction mixture, the extended primers will dissociate from the marker to form reaction products, excess primers will bind to the marker and to the reaction products and the process is repeated.

A reverse transcriptase PCR™ amplification procedure may be performed in order to quantify the amount of mRNA amplified. Methods of reverse transcribing RNA into cDNA are well known and described in Sambrook *et al.* (1989). Alternative methods for reverse transcription utilize thermostable, RNA-dependent DNA polymerases. These methods are described in Int. Pat. Appl. Publ. No. WO 90/07641 (specifically incorporated herein by reference). Polymerase chain reaction methodologies are well known in the art.

Another method for amplification is the ligase chain reaction ("LCR"), disclosed in EPA No. 320 308, and incorporated herein by reference in its entirety. In LCR, two complementary probe pairs are prepared, and in the presence of the target sequence, each pair will bind to opposite complementary strands of the target such that they abut. In the presence of a ligase, the two probe pairs will link to form a single unit. By temperature cycling, as in PCR™, bound ligated units dissociate from the target and then serve as "target sequences" for ligation of excess probe pairs. U.S. Patent 4,883,750 describes a method similar to LCR for binding probe pairs to a target sequence.

Qβ Replicase (QβR), described in Int. Pat. Appl. No. PCT/US87/00880, incorporated herein by reference, may also be used as still another amplification method in the present invention. In this method, a replicative sequence of RNA that has a region complementary to that of a target is added to a sample in the presence of an RNA polymerase. The polymerase will copy the replicative sequence that can then be detected.

An isothermal amplification method, in which restriction endonucleases and ligases are used to achieve the amplification of target molecules that contain nucleotide 5'-[α-thio]-

triphosphates in one strand of a restriction site may also be useful in the amplification of nucleic acids in the present invention.

Strand Displacement Amplification (SDA), described in U. S. Patent Nos. 5,455,166, 5,648,211, 5,712,124 and 5,744,311, each incorporated herein by reference, is another method of carrying out isothermal amplification of nucleic acids which involves multiple rounds of strand displacement and synthesis, *i.e.*, nick translation. A similar method, called Repair Chain Reaction (RCR), involves annealing several probes throughout a region targeted for amplification, followed by a repair reaction in which only two of the four bases are present. The other two bases can be added as biotinylated derivatives for easy detection. A similar approach is used in SDA. Target specific sequences can also be detected using a cyclic probe reaction (CPR). In CPR, a probe having 3' and 5' sequences of non-specific DNA and a middle sequence of specific RNA is hybridized to DNA that is present in a sample. Upon hybridization, the reaction is treated with RNase H, and the products of the probe identified as distinctive products that are released after digestion. The original template is annealed to another cycling probe and the reaction is repeated.

Still another amplification methods described in GB Application No. 2 202 328, and in Int. Pat. Appl. No. PCT/US89/01025, each of which is incorporated herein by reference in its entirety, may be used in accordance with the present invention. In the former application, "modified" primers are used in a PCR™-like, template- and enzyme-dependent synthesis. The primers may be modified by labeling with a capture moiety (*e.g.*, biotin) and/or a detector moiety (*e.g.*, enzyme). In the latter application, an excess of labeled probes is added to a sample. In the presence of the target sequence, the probe binds and is cleaved catalytically. After cleavage, the target sequence is released intact to be bound by excess probe. Cleavage of the labeled probe signals the presence of the target sequence.

Other nucleic acid amplification procedures include transcription-based amplification systems (TAS), including nucleic acid sequence based amplification (NASBA) and 3SR Gingeras *et al.*, Int. Pat. Appl. Publ. No. WO 88/10315, incorporated herein by reference. In NASBA, the nucleic acids can be prepared for amplification by standard phenol/chloroform extraction, heat denaturation of a clinical sample, treatment with lysis buffer and minispin columns for isolation of DNA and RNA or guanidinium chloride extraction of RNA. These amplification techniques involve annealing a primer that has target specific sequences. Following polymerization, DNA/RNA hybrids are digested with RNase H while double stranded DNA molecules are heat denatured again. In either case the single stranded DNA is made fully double stranded by addition of second target specific primer, followed by

polymerization. The double-stranded DNA molecules are then multiply transcribed by an RNA polymerase such as T7 or SP6. In an isothermal cyclic reaction, the RNA's are reverse transcribed into single stranded DNA, which is then converted to double stranded DNA, and then transcribed once again with an RNA polymerase such as T7 or SP6. The resulting products, whether truncated or complete, indicate target specific sequences.

Davey *et al.*, EPA No. 329 822 (incorporated herein by reference in its entirety) disclose a nucleic acid amplification process involving cyclically synthesizing single-stranded RNA ("ssRNA"), ssDNA, and double-stranded DNA (dsDNA), which may be used in accordance with the present invention. The ssRNA is a template for a first primer oligonucleotide, which is elongated by reverse transcriptase (RNA-dependent DNA polymerase). The RNA is then removed from the resulting DNA:RNA duplex by the action of ribonuclease H (RNase H, an RNase specific for RNA in duplex with either DNA or RNA). The resultant ssDNA is a template for a second primer, which also includes the sequences of an RNA polymerase promoter (exemplified by T7 RNA polymerase) 5' to its homology to the template. This primer is then extended by DNA polymerase (exemplified by the large "Klenow" fragment of *E. coli* DNA polymerase I), resulting in a double-stranded DNA ("dsDNA") molecule, having a sequence identical to that of the original RNA between the primers and having additionally, at one end, a promoter sequence. This promoter sequence can be used by the appropriate RNA polymerase to make many RNA copies of the DNA. These copies can then re-enter the cycle leading to very swift amplification. With proper choice of enzymes, this amplification can be done isothermally without addition of enzymes at each cycle. Because of the cyclical nature of this process, the starting sequence can be chosen to be in the form of either DNA or RNA.

Miller *et al.*, Int. Pat. Appl. Publ. No. WO 89/06700 (incorporated herein by reference in its entirety) disclose a nucleic acid sequence amplification scheme based on the hybridization of a promoter/primer sequence to a target single-stranded DNA ("ssDNA") followed by transcription of many RNA copies of the sequence. This scheme is not cyclic, *i.e.*, new templates are not produced from the resultant RNA transcripts. Other amplification methods include "RACE" and "one-sided PCR™" (Frohman, 1990, specifically incorporated herein by reference).

Methods based on ligation of two (or more) oligonucleotides in the presence of nucleic acid having the sequence of the resulting "di-oligonucleotide," thereby amplifying the di-oligonucleotide, may also be used in the amplification step of the present invention.

Following any amplification, it may be desirable to separate the amplification product from the template and the excess primer for the purpose of determining whether specific

amplification has occurred. In one embodiment, amplification products are separated by agarose, agarose-acrylamide or polyacrylamide gel electrophoresis using standard methods (see *e.g.*, Sambrook *et al.*, 1989).

Alternatively, chromatographic techniques may be employed to effect separation. There are many kinds of chromatography which may be used in the present invention: adsorption, partition, ion-exchange and molecular sieve, and many specialized techniques for using them including column, paper, thin-layer and gas chromatography.

Amplification products must be visualized in order to confirm amplification of the marker sequences. One typical visualization method involves staining of a gel with ethidium bromide and visualization under UV light. Alternatively, if the amplification products are integrally labeled with radio- or fluorometrically-labeled nucleotides, the amplification products can then be exposed to x-ray film or visualized under the appropriate stimulating spectra, following separation.

In one embodiment, visualization is achieved indirectly. Following separation of amplification products, a labeled, nucleic acid probe is brought into contact with the amplified marker sequence. The probe preferably is conjugated to a chromophore but may be radiolabeled. In another embodiment, the probe is conjugated to a binding partner, such as an antibody or biotin, and the other member of the binding pair carries a detectable moiety.

In one embodiment, detection is by Southern blotting and hybridization with a labeled probe. The techniques involved in Southern blotting are well known to those of skill in the art and can be found in many standard books on molecular protocols. See Sambrook *et al.*, 1989. Briefly, amplification products are separated by gel electrophoresis. The gel is then contacted with a membrane, such as nitrocellulose, permitting transfer of the nucleic acid and non-covalent binding. Subsequently, the membrane is incubated with a chromophore-conjugated probe that is capable of hybridizing with a target amplification product. Detection is by exposure of the membrane to x-ray film or ion-emitting detection devices.

One example of the foregoing is described in U. S. Patent No. 5,279,721, incorporated by reference herein, which discloses an apparatus and method for the automated electrophoresis and transfer of nucleic acids. The apparatus permits electrophoresis and blotting without external manipulation of the gel and is ideally suited to carrying out methods according to the present invention.

5. EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

5.1 EXAMPLE 1 – CONSTRUCTION OF VECTORS AND EXPRESSION IN TARGET CELLS

5.1.1 rAAV-RIBOZYME CONSTRUCTS

Recombinant AAV constructs were based on the pTR-UF2 vector (Zolotukhin *et al.*, 1996). They resemble the vector used by Flannery *et al.* (1997) to direct GFP expression to rat photoreceptors except that a 691-bp fragment of the proximal bovine rod opsin promoter replaced the 472-bp murine rod opsin promoter and the ribozyme gene replaced the *gfp* gene. The bovine promoter fragment contains three proximal promoter elements and the endogenous transcriptional start site at its 3N end (DesJardin and Hauswirth, 1996) and supports high efficiency, rat photoreceptor-specific expression *in vivo*. Active and inactive ribozymes were designed, tested and cloned as described above. Each ribozyme gene was followed by an internally cleaving hairpin ribozyme derived from plasmid pHC (Altschuler *et al.*, 1992) resulting in ribozyme cassettes of 140-152 bp. Self cleavage at the internal cutting site in the primary ribozyme RNA leaves identical 3N ends on each mature ribozyme. The ribozyme cassette was preceded by an intron derived from SV40 and followed by a polyadenylation signal in order to promote nuclear export of the ribozyme. Recombinant AAV titers were determined using both an infectious center assay (Flannery *et al.*, 1997) and a DNAse resistant physical particle assay employing a quantitative, competitive PCR of the *neo*^{r} gene contained within all rAAV-ribozyme particles (Zolotukhin *et al.*, 1996). Each of the four rAAV-ribozyme virus preparations contained 10^{10} to 10^{11} DNASE resistant particles per ml and 10^8 to 10^9 infectious center units per ml. Contaminating helper adenovirus and wild-type AAV, assayed by serial dilution cytopathic effect or infectious center assay respectively, were less than five orders of magnitude lower than rAAV.

5.1.2 Subretinal Injection of RAAV

Line 3 albino transgenic rats (P23H-3) on an albino Sprague-Dawley background (produced by Chrysalis DNX Transgenic Sciences, Princeton, NJ) were injected at the ages of P14 or P15. Animals were anesthetized by ketamine/xylazine injection, and a direction and b-waves were measured from the cornea-negative peak to the major cornea-positive peak. For quantitative comparison of differences between the two eyes of rats, the values from all the stimulus intensities were averaged for a given animal.

5.1.3 Retinal Tissue Analysis

Rats were euthanized by overdose of carbon dioxide inhalation and immediately perfused intracardially with a mixture of mixed aldehydes (2% formaldehyde and 2.5% glutaraldehyde). Eyes were removed and embedded in epoxy resin, and 1 μm thick histological sections were made along the vertical meridian. Tissue sections were aligned so that the ROS and Müller cell processes crossing the inner plexiform layer were continuous throughout the plane of section to assure that the sections were not oblique, and the thickness of the ONL and lengths of RIS and ROS were measured as described by Faktorovich *et al.* (1990). Briefly, 54 measurements of each layer or structure were made at set points around the entire retinal section. These data were either averaged to provide a single value for the retina, or plotted as a distribution of thickness or length across the retina. The greatest 3 contiguous values for ONL thickness in each retina were also compared to determine if any region of retina (*e.g.*, nearest the injection site) showed proportionally greater rescue; although most of these values were slightly greater than the overall mean of all 54 values, they were no different from control values than the overall mean. Thus, the overall mean was used in the data cited, since it was based on a much larger number of measurements.

5.1.4 RT-PCR™

For quantification of opsin mRNA retina from ribozyme injected or control eyes, retina were isolated without fixation and total RNA immediately extracted using the RNeasy Minikit (Qiagen, Santa Clarita, CA). RT-PCR™ was performed using the Pharmacia First-Strand cDNA synthesis kit employing oligo dT as the primer. Wild-type and transgene opsin cDNAs were amplified using a three primer system described above. Primers specific for β-actin cDNA (Timmers *et al.*, 1993) were included in each reaction for internal standardization.

5.2 EXAMPLE 2 – ENDOTHELIAL CELL PROLIFERATION IN RESPONSE TO ADENOSINE ANALOGUES

The subtype of adenosine receptor (A_{2B}) that mediates the proliferative effect of adenosine on HREC was determined by the following studies. The non-selective adenosine receptor agonist NECA, after 48 hr of exposure, induced a concentration-dependent increase of DNA synthesis in HRECs, as indicated by bromodeoxyuridine (BrdU) incorporation. In contrast, neither the A_{2A} adenosine receptor agonist CGS21680 (2-p-(2-carboxyethyl) phenethylamino-5'-N-ethylcarboxamidoadenosine) at concentrations ranging from 10 nM to 10 μM, nor the A_1 adenosine receptor agonist CPA (N^6-cyclopentyladenosine) at concentrations ranging from 10 nM to 10 μM increased BrdU incorporation by HRECs. The addition of the adenosine receptor antagonist XAC (xanthine amine congener) at 10 μM completely prevented the NECA-stimulated BrdU incorporation. In contrast, neither the selective A_1 adenosine receptor antagonist CPX (8-cyclopentyl-1,3-dipropylxanthine) at 20 nM, nor the selective A_{2A} adenosine receptor antagonist SCH58261 (5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo(4,3-E)-1,2,4-trizolo(1,5-*c*) pyrimidine) at 60 nM attenuated the stimulatory effect of NECA on BrdU incorporation by HRECs. These findings indicate that the proliferative effects of NECA are mediated by the A_{2B} adenosine receptor in HREC in culture (Table 3).

TABLE 3
EFFECT OF ADENOSINE RECEPTOR AGONISTS AND ANTAGONISTS ON HREC (FOLD-CHANGE *VS.* UNTREATED)

	Proliferation		VEGF		CAMP Content
	Cell Count	BrdU Incorporation	Protein	MRNA	
Untreated	1.0	1.0	1.0	1.0	1.0
CGS(1)	1.1 ± 0.03	1.0 ± 0.08	1.3 ± 0.20	0.8 ± 0.37	1.1 ± 1.02
CPA (10 μm)	0.9 ± 0.04	0.8 ± 0.06	1.1 ± 0.27	1.1 ± 0.22	(ND)
NECA (10 μm)	1.6* ± 0.03	1.5* ± 0.05	2.7* ± 0.31	4.2* ± 0.65	11.6* ± 2.36
NECA + CPX (20 nM)	1.5* ± 0.03	1.5* ± 0.05	3.2* ± 0.45	3.8* ± 1.14	9.9* ± 4.01
NECA + SCH(2) (60 nM)	1.6* ± 0.04	1.5* ± 0.04	3.2* ± 0.23	3.6* ± 0.16	7.8* ± 2.13
NECA + XAC (10 μm)	1.0 ± 0.03	0.9 ± 0.06	0.9 ± 0.18	0.7 ± 0.26	0.8 ± 1.18

TABLE 3 (con't)

	Proliferation		VEGF		CAMP Content
	Cell Count	BrdU Incorporation	Protein	MRNA	
[NECA + Enprofylline (10 μm)	1.1 ± 0.04	(ND)	(ND)	(ND)	(ND)
NECA + JW V108 (10 μm)	1.0 ± 0.02	(ND)	(ND)	(ND)	(ND)]

(ND) = specific condition not tested.
(1) Full compound name is CGS21680.
(2) Full compound name is SCH58261.
* Significantly different from untreated ($p < 0.005$).

The data for cell counts were consistent with those for BrdU incorporation. Treatment with NECA for 48 hr resulted in a concentration-dependent increase in HREC number, whereas neither CGS21680 nor CPA caused an increase in cell number. Of the three adenosine receptor antagonists tested, only XAC significantly inhibited the increase in cell number induced by 10 μM NECA (Table 3). These compounds, like XAC, blocked the proliferative effects of NECA as measured by cell counts, supporting that the proliferative effects of NECA on HREC are mediated by the adenosine A_{2B} receptor (Table 3).

5.2.1 cAMP ACCUMULATION

Further evidence for the presence of the A_{2B} adenosine receptors in HRECs was obtained by determining the cAMP content of intact HRECs following treatment with adenosine receptor agonists and antagonists (Grant *et al.*, 1999). The cAMP content of intact HRECs following treatment with adenosine receptor agonists and antagonists was next examined. NECA increased cAMP content of HRECs in a concentration-dependent manner, with an EC_{50} value of 24 μM. In contrast, the selective high affinity A_{2A} adenosine receptor agonist CGS 21680 (at concentrations up to 100 μM) had no significant effect on cAMP content of HREC. The effect of selective A_1 and A_{2A} adenosine receptor antagonists on NECA-induced accumulation of cAMP was also examined. NECA (10 μM)-induced increase in cAMP content in HRECs was not signficantly inhibited by either the selective A_{2A} adenosine receptor antagonist SCH58261 or by the selective A_1 adenosine receptor antagonist CPX. On the other hand, the non-selective adenosine receptor antagonist XAC completely blocked the effect of NECA on cAMP accumulation, support that activation of the adenosine A_{2B} receptor was

required for cAMP release in HREC (Table 3). These data indicate that the proliferative effects of NECA were mediated through the A_{2B} receptor subtype.

5.2.2 VEGF CONFIRMED AS THE MEDIATOR OF ADENOSINE'S VASOPROLIFERATIVE EFFECT

Incubation with VEGF resulted in BrdU incorporation to a level approximating that induced by normal growth medium. The VEGF antibody significantly reduced DNA synthesis induced by VEGF. Incubation with NECA increased DNA synthesis to levels comparable to that induced by normal growth medium. The addition of VEGF antibody resulted in a decrease in NECA-induced BrdU incorporation, which was statistically significant at the highest tested concentration of antibody (FIG. 2A). Similar results were observed at either 24 or 48 hr of exposure to the test agents.

Antisense oligonucleotides for both A_{2B} adenosine receptor and VEGF caused a significant decrease in VEGF in the conditioned medium following NECA exposure (FIG. 2B). This effect was most pronounced for the receptor antisense oligonucleotide with 10 nM NECA, but was evident for all concentrations of NECA tested. The VEGF antisense oligonucleotide also caused a decrease in secreted VEGF in response to NECA, albeit not to the same magnitude as that observed with the A_{2B} adenosine receptor antisense.

NECA induces a dose-dependent increase in ERK activation at 5 and 10 min in HRECs. Both A_{2B} antagonists Enprofylline and JW V-108 abolished ERK activation by NECA. While NECA activated ERK, the A_{2A} agonist, CGS (high doses, 1-10 μM) reduced the basal ERK levels. CGS may be activating cAMP and this cAMP response may down regulate ERK activation. These data support a role for adenosine in the activation of ERK that may then induce the phosphorylation of HIF-1α.

5.2.3 ADENOSINE A_{2B} RECEPTOR ACTIVATION REQUIRED FOR HREC CHEMOTAXIS

The role of the A_{2B} receptor was further characterized by examining the effects of the A_{2B} antagonists JW V108 and Enprofylline on HREC chemotaxis, capillary tube formation and signal transduction pathways following stimulation with the adenosine analogue NECA. NECA induced HREC chemotaxis in a concentration-dependent manner that was inhibited by Enprofylline and JW V108.

5.2.4 ADENOSINE A_{2B} RECEPTOR REQUIRED FOR HREC ERK ACTIVATION

NECA (1 nmol/L – 10 μmol/L) induced a transient activation of ERK which peaked at 5 min and desensitized within 20 min. The rate of desensitization was dependent on NECA concentration since higher doses of NECA produced a more rapid desensitization (FIG. 3). The A_1-selective agonist CPA was also capable of stimulating ERK (FIG. 4), however the A_{2A}-selective agonist CGS did not activate ERK. In order to determine the intracellular signaling pathways activated by NECA that regulate ERK activity, we pretreated cells for 30 min with the ERK/MPAK kinase (MEK) inhibitor PD98059 or the PKA inhibitor H-89 and stimulated with NECA for 5 min. PD98059 abolished ERK activation, while H-89 increased basal ERK activation (FIG. 5). H-89 did not block NECA-stimulated ERK activation, suggesting that PKA is not involved in signaling from the adenosine receptor to ERK. The non-selective adenosine receptor antagonist XAC decreased ERK activation by high concentrations of NECA, but modestly increased ERK activation in control conditions and in response to 1 and 10 nM NECA. Interestingly, prolonged activation with NECA in the presence of XAC or SCH and CPX reduced the rate of ERK desensitization, suggesting that adenosine receptors are involved in both activation and desensitization of ERK.

Phosphorylation of cAMP response element binding protein (CREB) at Ser^{133} was examined following NECA stimulation in order to determine whether activation of cAMP pathways by NECA occurred independently of ERK activation. Cells were pretreated with PD98059 or H-89 and assayed for active CREB by western blot. PD98059 did not alter CREB activation; however both H-89 and XAC blocked CREB phosphorylation. These data indicate that ERK activation by NECA occurs independently of the cAMP response (FIG. 5).

Enprofylline and JW V108 exhibit greater selectivity for the A_{2B} receptor. Cells were pre-treated with both antagonists for 10 min and stimulated with increasing concentrations of NECA. Enprofylline completely abolished ERK activation, while JW V108 inhibited ERK activation at all concentrations except for 10 μM. These data suggest that ERK activation occurs through both the A_{2B} and A_1 receptors, but not the A_{2A} receptor (FIG. 6). These data support a role for adenosine in the activation of ERK that may then induce the phosphorylation of HIF 1-α.

5.3 EXAMPLE 3 – DEVELOPMENT AND TESTING OF RIBOZYME TARGETING A_{2B} ADENOSINE RECEPTOR MRNA

The cleavage site of the A_{2B} antisense, between nucleotides 183 and 184, was demonstrated to be accessible within the secondary structure of the native mRNA by the

antisense studies. A hammerhead ribozyme designed to cleave this message was then synthesized along with a 14-nucleotide target sequence (FIG. 7). This target was end-labeled in a standard kinase reaction with ^{32}P, then incubated along with ribozyme (1:1 molar ratio) for 1, 2, 3, 4, 5, 10, 30, 60, 120 and 180 min. Nearly 90% of target was cleaved by 60 min (FIG. 7), demonstrating the efficacy and rapid action of this ribozyme in a cell-free assay system. The ribozyme's effects on HREC proliferation and VEGF synthesis in response to adenosine receptor activation were examined. HRECs were plated in serum-free medium overnight to adhere and make them quiescent. Unattached cells were then removed by washing with Hank's balanced salt solution (HBSS). The cells were then incubated with 1 U/mL adenosine deaminase (ADA) for 20 min, after which was added either medium alone, 1 μmol/L A_{2B} receptor ribozyme, or 1 μmol/L of a synthetic mixed oligonucleotide of the same length as the ribozyme, all of which contained 10 μmol/L NECA. Cells were then incubated for a total of seven days. Sampling occurred every 24 hr as follows. Conditioned medium was collected and stored at –70°C until the end of the assay, after which it was analyzed for VEGF using a commercially available ELISA. The cells were enzymatically dissociated from the wells and counted using a Coulter counter. These latter results were then used to normalize the VEGF data to a constant cell number. FIG. 8 shows that cells treated with ribozyme express up to 60% less VEGF protein in response to NECA than do either untreated cells or cells treated with sham oligonucleotide. Similarly, these same cells exhibited a 50% reduction in proliferation 7 days after NECA stimulation when exposed to ribozyme compared to control.

5.3.1 Oxygen-Induced Retinal Neovascularization in the Neonatal Mouse

The potential efficacy of administering adenosine receptor antagonists to reduce retinal neovascularization brought on by ischemic insult was examined. In the neonatal mouse model of oxygen-induced retinopathy, 7-day-old mice are placed with their nursing dams in a 75% oxygen atmosphere for 5 days. Upon return to normal air, these mice develop retinal neovascularization, with peak development occurring 5 days after their return to normoxia. During this time, the animals receive by daily intaperitoneal injection a pharmacologically relevant concentration of adenosine receptor antagonist or vehicle (0.15% vol./vol. DMSO in normal saline) alone. Exemplary antagonists tested include XAC and 3-n-propylxanthine (Enprofylline), and JW V108, both at a concentration of 30 mg/Kg of body weight.

At the fifth day after return to normoxia, the animals were sacrificed and the eyes removed for fixation in sodium cacodylate buffered acrolein (5% vol./vol.). After extensive washing, the eyes were embedded in epoxy resin for sectioning. At least twenty sections, 1 μm

thick with 5 μm between sections, were cut sagitally from each eye, resulting in a total sampling thickness of 120 μm for each eye. Sections were then stained to visualize cell nuclei.

Individuals masked to the identity of treatment counted all cell nuclei above the inner limiting membrane for all 20 sections from each eye. These data were then expressed as the sum of the counts from each eye. The efficacy of treatment with a particular antagonist was then calculated as the fraction of total nuclei in antagonist or vehicle treatment over total nuclei in uninjected control. FIG. 9 summarizes these findings and shows that 3 adenosine receptor antagonists tested inhibit oxygen-induced retinal neovascularization by 54% for XAC and 70% for Enprofylline. Selected animals were anesthetized and then perfused with 10 mL 50 mg/mL fluorescein-Dextran 2,000,000 in formaldehyde (4%) *via* cardiac puncture. Whole retinas from these eyes were then flat-mounted for qualitative assessment of retinal neovascularization by fluorescence microscopy.

5.3.2 LASER-INDUCED VENOUS THROMBOSIS IN MOUSE EYES

Pilot studies were performed to determine the feasibility of inducing retinal neovascularization in the mouse eye by occluding each branch vein *via* laser photocoagulation as described. There occurs preretinal neovascularization as a result of profound retinal ischemia. The presence of corneal and iris neovascularization also supports the presence of severe retinal ischemia with the release of growth factors into the vitreous that ultimately reach the cornea and cause neovascularization.

The ability to differentiate the source of endothelial cells responsible for retinal vasculogenesis following photodynamic venous thrombosis can be accomplished using the NOD.B10$^{B6.gfp}$ chimeric mouse. Re-infiltration of vascular areas by bone marrow-derived, nucleus-containing cells from reconstituted bone marrow in the NOD.B10$^{B6.gfp}$ mouse results in easy detection of bone marrow-derived cells by virtue of their expressing *gfp*. This demonstrates the feasibility of examining retinal vasculature for *gfp* expression as an indicator of newly formed vessels following photodynamic venous coagulation and/or treatment with adenosine analogues to stimulate endothelial cell migration and proliferation.

5.4 EXAMPLE 4 – BLOOD ANALYSIS FOR CD34$^+$ PROGENITOR CELLS IN DIABETIC PATIENTS WITH PDR

CD34$^+$ progenitor cells were isolated from the leukocyte fraction of blood from two patients who were experiencing rapid deterioration of their vision associated with new onset retinal neovascularization. These samples were analyzed by flow cytometry. The number of

$CD34^+$ cells detected in the serum of one patient was 30-fold higher than those detected in a non-diabetic control patient sample. The second patient was 15-fold higher than non-diabetic control levels. This intriguing finding is being pursued as part of a multicenter study involving patients with severe NPDR and will not be pursued here. This observation does, however, establish the justification for examining the correlation between circulating angioblasts and IGF-I/VEGF serum levels.

5.4.1 Production of Bone Marrow Chimeric Mice

C57BL/6-*gfp* transgenic mice were maintained through selected brother-sister matings. Because homozygosity at the *gfp*-transgene is lethal at day E14 of fetal development, breeding pairs are established consisting of C57BL/6-$gfp^{-/-}$ males and C57BL/6-$gfp^{+/-}$ females. The offspring from such breeding pairs are approximately 50% non-fluorescent and 50% fluorescent within both sexes. This represents an ideal situation for the production of bone marrow chimeras since gfp^+ bone marrow can be introduced into syngeneic gfp^- (non-fluorescent) siblings. Production of C57BL/6-*gfp* bone marrow chimeras were carried out as described previously by the Co-PI (LaFace and Peck, 1989).

5.4.2 HIF-1α Levels in Response to Adenosine

Mouse monoclonal antibodies (IgG) which recognize the C-terminus of HIF-1α were obtained from Novus Biologicals. This reagent is used to monitor stability and phosphorylation of HIF-1α following treatment of HREC with the non-specific adenosine receptor agonist NECA at concentrations of 10 nM – 10 μM (Richard *et al.*, 1999). HREC is exposed to varying O_2 concentrations (1, 3, 10%) to induce hypoxia and will be used for comparison to NECA treated cells. After treatment, cells will be lysed in a buffer containing Triton X-100, 100 mM NaCl and a combination of proteinase and phosphatase inhibitors as previously described (Davis *et al.*, 1999). Protein concentration is determined by the BCA assay and 5-10 μg of nuclear extract was separated by electrophoresis on 8% SDS polyacrylamide gels. Proteins are electrophoretically blotted to PDVF and incubated with a 1:1000 dilution of antibodies to HIF-1α. The presence of immunoreactivity is detected by enhanced chemiluminscence (ECL, Amersham) for qualitative experiments. To confirm that slowly migrating forms correspond to phosphorylated HIF-1α, extracts are treated with phosphatase (lambda phosphatase, NEB) in the absence of phosphatase inhibitors prior to electrophoresis (Forsythe *et al.*, 1996). In the next series of experiments, 5-10 μg of nuclear extract is incubated with a ^{32}P-labeled 47-bp double-stranded DNA probe based on the HRE of the VEGF gene. Competition experiments include

excess unlabeled oligonucleotide. In this assay, a dose-dependent increase in the amount of shifted VEGF probe following treatment of cells with NECA is expected. This shift is competed by unlabeled hypoxia response element (HRE) DNA based on the VEGF or erythropoietin promoter.

5.4.3 INVOLVEMENT OF HYPOXIA RESPONSE ELEMENT IN RESPONSE TO ADENOSINE

The adenosine-responsive region of the VEGF promoter is analyzed in a manner similar to Forsythe *et al.* who mapped the hypoxia response element (HRE) of the VEGF gene upstream of the transcription initiation site (Forsythe *et al.*, 1996). A 1.6 kb fragment of VEGF genomic DNA that contains 1.2 kb of 5' flanking sequence and 0.4 kb of primary transcript was ligated into the luciferase-reporter plasmid pGL2 (Promega). This plasmid contains an intron and polyadenylation signal from SV40 but lacks proximal promoter and enhancer elements. The promoter dissection by Forsythe *et al.* (1996) as well as others (Shima *et al.*, 1996) relied on convenient restriction sites to generate promoter deletions, but an inverse PCR™ technique (Hemsley *et al.*, 1989) may be used to generate a set of internal deletions of the VEGF promoter. Because primers can be placed with precision, PCR™-generated deletion permits better discrimination of closely spaced promoter elements than methods that depend on timed exonuclease reactions (Xu and Gong, 1999). Selected regions of the VEGF promoter were deleted using divergent primers and a commercial PCR™ mix designed to promote long-range PCR™. The PCR™ product was then circularized by ligation. To avoid PCR™-generated mutations elsewhere in the plasmid (especially in the luciferase gene), a second set of primers was added to amplify the deleted promoter region, which was then re-cloned in the reporter plasmid. The upstream regions were sequenced to exclude unintentional PCR™ mutagenesis.

Human retinal endothelial cells are maintained in culture as previously described (Grant *et al.*, 1999). Based on the DEAE-Dextran method (Agarwal *et al.*, 1998; Selden, 1993), HRECs may be grown to about 50% confluence prior to transfection with 1.5 ml DEAE-Dextran solution/100 mm plate containing 5.0 μg DNA of the vectors. The cells are washed twice with Tris-buffered saline and media containing 10% NuSerum is added to the plates. The transfection solution is then added to each dish drop by drop equally over each portion of the plate and then gently swirled. To increase transfection efficiency, chloroquine diphosphate (100 μM) is added to the medium at this stage, and cells are incubated for 4 hr at 37°C in 5% CO_2/room air. The cells are shocked for 1 min at room temperature by the addition of 10% DMSO in PBS, washed with PBS, then chloroquine free medium is added to each plate. In addition to the VEGF-luciferase plasmid, cells are co-transfected with pSVbgal, to serve as a

measure of transfection efficiency. Cells from duplicate transfections are allowed to recover for 24 hr in 6-well plates (Costar). Cells are then given fresh medium containing 10 nM - 10 μM NECA or vehicle, and incubated a further 30 min to 4 hr in 5% CO_2, 95% air at 37°C during which time luciferase activity is measured using a fluorescence microplate reader. β-galactosidase activity is measured by hydrolysis of 2-nitrophenyl-β-D-galactopyranoside. Relative luciferase activity is calculated as light units produced normalized to β-galactosidase activity and protein concentration on cell lysates as measured by BCA assay (Pierce).

5.4.4 Pharmacological Analysis of Adenosine-Stimulated VEGF Expression

Previously it was demonstrated that NECA stimulates VEGF expression through the A_{2B} receptor (Grant *et al.*, 1998). These experiments relied on selectively blocking A_1 and A_{2A} receptors and determining the A_{2B} component by subtracting the A_1 and A_{2A} responses. Since this study was published, selective A_{2B} antagonists have been obtained that also block NECA-stimulated ERK activation. These experiments support the role of the A_{2B} receptor in NECA-stimulated proliferation; however the intracellular signal transduction pathways from the receptor to the nucleus and VEGF promoter have not been defined. The A_{2B} receptor has been shown to signal through two separate G-protein-coupled signal transduction pathways in several cell types (D'Angelo *et al.*, 1997; Wu *et al.*, 1993; Cook and McCormick, 1993). The "classical" A_{2B} pathway activates adenylyl cyclase through Gs, while recent evidence suggests that the A_{2B} receptor also couples through Gq/11 to activate phospholipases and PKC (D'Angelo *et al.*, 1997; Wu *et al.*, 1993; Cook and McCormick, 1993). In order to define transcription factors that regulate VEGF expression, it is necessary to define the NECA-stimulated signal transduction pathways.

HRECs transfected with the luciferase/HRE constructs (described above) are assayed in a 96-well plate format using a temperature-controlled microplate luminometer as described above. Cells are assayed at 1 hr intervals and are assayed with activators and inhibitors under hypoxic and normoxic conditions. Negative controls contain DMSO or PBS at the concentration used as a vehicle. Plates are sealed in a chamber under the appropriate oxygen concentration and 10 mM HEPES is added to the assay medium to maintain pH. In experiments using NECA as an activator, the assay is performed as a dose-response (10 nM to 10 μM) in the presence of inhibitors and in the presence and absence of the A_{2B} receptor antagonists, Enprofylline and JW V108. Inhibitors are used at 10 times the inhibition constant. In experiments where activators are being used (*e.g.*, forskolin, 8-Br-cAMP, phorbol esters), luciferase activity is assayed as a dose response to the activator. The role of AC and PKA in

VEGF regulation is determined by pre-treating cells with cholera toxin, forskolin, H-89 and 8-Br-cAMP. If A_{2B}-stimulation of the AC/cAMP/PKA pathway is responsible for NECA-stimulated VEGF expression, then cholera toxin and 8-Br-cAMP and forskolin should increase VEGF promoter activity in the absence of NECA, while H-89 and 8-Br-Rp-cAMP should block NECA-stimulated luciferase activity if transcription is mediated by cAMP-dependent pathways. However, it is possible that A_{2B} coupling through members of the Gq/11 family is responsible for NECA-stimulated VEGF transcription. The hypothesis may be tested by stimulating the cells with NECA and selectively inhibiting this pathway at multiple points. The Gq-selective antagonist GP Antagonist 2A (Mukai *et al.*, 1992) will be used to block signaling at the level of receptor coupling. Because Gq can activate two initially divergent pathways, both intracellular Ca^{++} chelators BAPTA/AM and PKC inhibitors (Go 6976 for Ca^{++} dependent PKC isoforms, Calphostin C as a general inhibitor) are tested for their ability to block VEGF induction stimulated by NECA. Similarly, the PKC activator PMA (phorbol 12-myristate-13-acetate) and thapsagargin (which increases intracellular Ca^{++} by inhibiting the endoplasmic reticulum Ca^{++}-ATPase) are tested for their ability to substitute for NECA in stimulating luciferase activity. If VEGF induction is due to A_{2B} activation of PKC or increased intracellular calcium, then PMA and thapsagargin, respectively, will substitute for NECA. Finally, the ERK (MEK) inhibitor PD98059 will be added to experiments showing increased VEGF expression in order to determine the role of ERK in VEGF induction.

5.4.5 Western Analysis of HIF-1α

Cells are lysed in a buffer containing Triton X-100®, 100 mM NaCl and a combination of proteinase and phosphatase inhibitors as previously described (Davis *et al.*, 1999). Protein concentration is determined using the BCA assay. Five-ten μg of nuclear extract is separated by electrophoresis on 8% SDS polyacrylamide gels. In the first series of experiments, proteins are electrophoretically blotted to PDVF and incubated with a 1:1000 dilution of antibodies to HIF-1α. The presence of immunoreactivity is detected by enhanced chemiluminscence (ECL, Amersham) for qualitative studies.

5.4.6 Preparation of Luciferase-Reporter Plasmids

A 363-bp fragment of the VEGF promoter has been cloned into the pGL2-Basic plasmid (Promega). The VEGF promoter was inserted upstream of firefly luciferase and drives the expression of this protein. This 336-bp fragment contains a 33-bp region encoding the hypoxia response element (HRE). This HRE may be deleted using inverse PCR™ as described

(Hemsley *et al.*, 1989) and this construct and the wild type version may be used to test the effect of NECA on the activation of the VEGF promoter.

5.4.7 TRANSFECTION OF HRECs

Based on the DEAE-Dextran method (Agarwal *et al.*, 1998; Selden, 1993), HRECs are grown to about 50% confluence prior to transfection with 1.5 ml DEAE-Dextran solution/100 mm plate containing 5.0 μg DNA of the vectors. The cells are washed twice with Tris-buffered saline and media containing 10% NuSerum is added to the plates. The transfection solution is then added to each dish drop by drop equally over each portion of the plate and then gently swirled. To increase transfection efficiency, chloroquine diphosphate (100 μM) is added to the medium at this stage. Cells are incubated for 4 hr at 37°C in 5% CO_2/room air. The cells are shocked for 1 min at room temperature by the addition of 10% DMSO in PBS, washed with PBS, then chloroquine free medium is added to each plate.

5.4.8 DUAL–LUCIFIERASE REPORTER ASSAY

Transfected cells are given fresh medium containing 10 nM – 10 μM NECA or vehicle, and incubated a further 30 min to 4 hr in 5% CO_2, 95% air at 37°C during which time luciferase activity is measured using a fluorescence microplate reader. β-galactosidase activity is measured by hydrolysis of 2-nitrophenyl-β-D-galactopyranoside. Relative luciferase activity is calculated as light units produced normalized to β-galactosidase activity and protein concentration on cell lysates as measured by BCA assay (Pierce).

5.4.9 PROMOTER STUDIES

Because primers can be placed with precision, PCR™-generated deletion permits better discrimination of closely spaced promoter elements than methods that depend on timed exonuclease reactions. The method of Xu and Gong (199) may be used. Selected regions of the VEGF promoter are deleted using divergent primers and a commercial PCR™ mix designed to promote long-range PCR™. The PCR™ product is then circularized by ligation. To avoid PCR™-generated mutations elsewhere in the plasmid (especially in the luciferase gene) a second set of primers is added to amplify the deleted promoter region, which is then re-cloned in the reporter plasmid. The upstream regions are sequenced to exclude unintentional PCR™-mutagenesis.

5.5 EXAMPLE 5 - DESIGN AND TESTING OF A_{2B} RECEPTOR-SPECIFIC RIBOZYMES

A hammerhead ribozyme was designed to cleave the mRNA for the A_{2B} receptor following nucleotide 183 (FIG. 6). This site was demonstrated to be accessible within the folded structure of the mRNA based on experiments using antisense oligodeoxynucleotides to inhibit expression of A_{2B} in tissue culture (Grant *et al.*, 1999).

Because the viral *rep* gene is missing from AAV vectors, site-specific integration does not occur, but the vector appears to randomly integrate into host DNA randomly (Kearns *et al.*, 1996; Ponnazhagan *et al.*, 1997). Recombinant AAV (rAAV) vectors have been used successfully in long-term gene delivery to the retina (Flannery *et al.*, 1997; Bennett *et al.*, 1997), the lung (Flotte *et al.*, 1993), muscle (Kessler *et al.*, 1996; Xiao and Samulski, 1996), brain (Klein *et al.*, 1998; Xiao *et al.*, 1997), spinal cord (Peel *et al.*, 1997), liver (Snyder *et al.*, 1997) and blood vessels (Rolling *et al.*, 1997). In particular, AAV infects vascular endothelial cells *in vivo* (Gnatenko *et al.*, 1997; Lynch *et al.*, 1997). A single intravitreal injection of AAV expressing marker proteins (β-galactosidase or *gfp*) was able to genetically transduce a variety of cell types in the guinea pig eye, including blood vessels, for up to one year post injection with no evidence of inflammation or other abnormalities (Guy *et al.*, 1999). Unlike adenovirus vectors used in gene therapy trials, AAV does not cause inflammation and does not provoke a cell-mediated immune response (Bennett *et al.*, 1997; Bennett *et al.*, 1999). Unlike retroviral vectors, AAV is able to infect non-cycling cells, such as vascular endothelial cells. Retroviral vectors have been used for dividing endothelial cells in culture, but not for non-dividing cells in vivo. The PGK1 HRE promoter may be used. As an alternative to the PGK1 promoter, the adenosine-responsive region of VEGF in conjunction with the SV-40 proximal promoter elements may be used in order to regulate expression of adenosine receptor ribozymes. In this way, an autologously regulating feedback-loop may be established to reduce the expression of the A_{2B} receptor.

5.5.1 ANIMALS

Male NOD.*Gfp*$^{-/-}$ mice are gamma-irradiated (650-850 R) and placed on acid water (pH 2.0). Between 4-6 hrs after irradiation, the recipient mice are reconstituted with bone marrow from male NOD.*Gfp*$^{-/+}$ mice (whole bone marrow isolated from the long bones of the front and hind legs of C57BL/6-*gfp*$^{-/+}$ mice) injected intravenously *via* the tail vein. Reconstitutions are carried out using 10^6-10^7 bone marrow cells per recipient. After reconstitution, the mice are observed daily for signs of wasting disease or other complications; however, survival approaches 100%. Successful reconstitution is determined by flow cytometric analysis of a

drop of blood. Leukocytes constitutively produce *gfp* (thus there is no dilution of fluorescence in daughter cells) while erythrocytes remain non-fluorescent.

The NOD.*Gfp* mouse line was derived from breeding female NOD mice with a C57BL/6-*gfp* transgenic male mouse. *Gfp*-positive offspring were backcrossed to NOD mice at each generation. Animals are followed closely for the onset of diabetes. Diabetic animals may receive one or more units of humulin NPH in the evening. Their blood sugars are measured once every two weeks or, if necessary, more frequently.

5.5.2 QUANTIFYING CIRCULATING ANGIOBLASTS

Mice are anesthetized deeply with ketamine/xylazine (70 mg/kg, 15 mg/kg, respectively) and exsanguinated by cardiac puncture. Low-density mononuclear cells (less than 1.077g/ml) are recovered by density centrifugation using Ficoll-Hypaque to enrich their numbers for subsequent analysis. Circulating angioblasts are enumerated using PE-conjugated anti-CD34 antibody staining. This allows differentiation of angioblasts from the total leukocyte population as well as quantification by two-channel flow cytometry. All leukocytes exhibit green fluorescence (*via gfp* expression), but only angioblasts exhibit concomitant red fluorescence (*via* specific Ab binding).

5.5.3 PREPARATION OF RETINAL WHOLE MOUNTS

Mice are anesthetized as described above, then perfused through the left ventricle with 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4. The eyes are enucleated and placed in 4% paraformaldehyde for 24 hr. The retinas are dissected from the globes and incised with four radial cuts to allow flat mounting with glycerol-gelatin. The flat mounted retinas are viewed by fluorescence microscopy and photographed. One thousand capillary cells are counted to determine the percentage which exhibit *gfp* expression.

5.5.4 THE C57BL/6-GFP TRANSGENIC MOUSE AND PRODUCTION OF NOD.B10$^{B6.GFP}$ CHIMERIC MICE

C57BL/6-gfp^{+} transgenic mice are maintained through selected brother-sister matings. Because homozygosity at the *gfp*-transgene is lethal, breeding pairs are established consisting of C57BL/6-$gfp^{-/-}$ males with C57BL/6-$gfp^{+/-}$ females (where C57BL/6-$gfp^{+/-}$ represents "near" homozygous mice). The offspring, then, are approximately 50% non-fluorescent and 50% fluorescent. This represents an ideal situation for the production of bone marrow chimeric mice since the gfp^{+} bone marrow can be introduced into syngeneic gfp^{-} (non-fluorescent) siblings.

Production of bone marrow chimeric mice may be carried out as detailed previously by LaFace and coworkers (1989). In brief, at time of hematopoietic stem cell reconstitution, young adult NOD.B10 mice (5-6 weeks of age) are gamma-irradiated (650-850 R) and placed on acid water (pH 2.0). Between 4-6 hrs after irradiation, the reconstituting cell population (whole bone marrow isolated from the long bones of the front and hind legs of C57BL/6-*gfp*$^+$) are injected into each host intravenously *via* the tail vein. Reconstitution is carried out using 10^6-10^7 bone marrow cells per recipient. After reconstitution, the mice are observed daily for signs of wasting disease or other complications, however, successful reconstitution typically approaches 100%.

5.5.5 IDENTIFICATION OF DIFFERENTIALLY EXPRESSED GENES DURING ISCHEMIA INDUCED ANGIOGENESIS

On days 2, 4, 7, 10 and 14 following laser treatment, mice are euthanized and their eyes removed. Retinas are removed and collected for analysis to determine temporal changes in gene expression that may lead to the recruitment of angioblasts and the development of preretinal neovascularization. Total RNA is isolated from each retina for use in microarrays and compared to the RNA obtained from the untreated eyes. If angioblasts are bone marrow-derived, as indicated by expression of *gfp*, then *gfp*-positive cells are collected from the preretinal tufts by laser capture using the Bio-Rad 1024ES Confocal Microscope. Total RNA is extracted from these captured cells and compared to RNA from neighboring retinal endothelial cells (resident cells) as well as from angioblasts obtained from peripheral blood as described above (Takahashi *et al.*, 1999). Gene expression for all of these RNA isolates is determined by cDNA microarray analysis.

TABLE 4

ILLUSTRATIVE HAIRPIN RIBOZYME TARGETS OF THE PRESENT INVENTION

RIBOZYME	SEQUENCE			SEQ ID NO:	REFERENCE
	Cleavage site				
	HelixII ↓		Helix I		
ROD OPSIN MRNA-SPECIFIC:					
P23L target:	acgc	a gcc	ucuucg-3'	SEQ ID NO:3	Berson *et al.*, 1991
Ribozyme arms:	ugcg	aaga	agaagc-5'		
F45L target:	acau	g guu	cugcug	SEQ ID NO:4	Sung *et al.*, 1991
Ribozyme arms:	ugug	aaga	gacgac		
G51A target:	ugcu	g gcc	uucccc	SEQ ID NO:5	Macke *et al.*, 1993
Ribozyme arms:	acgg	aaga	aagggg		
G51G target:	ugcu	g guc	uucccc	SEQ ID NO:6	Dryja *et al.*, 1991
Ribozyme arms:	acgg	aaga	aagggg		
P53R target:	gcug	g gcu	uccggc	SEQ ID NO:7	Inglehearn *et al.*, 1992
Ribozyme arms:	cgac	aaga	aggccg		

TABLE 4 (con't)

RIBOZYME	SEQUENCE Cleavage site HelixII		Helix I	SEQ ID NO:	REFERENCE
Q64stop target:	ucac	c guc	**u**agcac	SEQ ID NO:8	Macke *et al.*, 1993
Ribozyme arms:	agug	aaga	aucgug		
G90D target:	aggu	g **g**cu	ucacca	SEQ ID NO:9	Sieving *et al.*, 1992
Ribozyme arms:	uccg	aaga	aguggu		
G106W target	uuc**u**	g gcc	ccacag	SEQ ID NO:10	Sung *et al.*, 1991
Ribozyme arms:	aagg	aaga	gguguc		
G114D target:	ugga	g **ga**c	uucuuu	SEQ ID NO:11	Vaithinathan *et al.*, 1994
Ribozyme arms:	accu	aaga	aagaaa		
R135L target:	aucg	a **guu**	guacgu	SEQ ID NO:12	Jacobson *et al.*, 1991
Ribozyme arms:	uagc	aaga	caugca		

TABLE 4 (con't)

RIBOZYME	SEQUENCE Cleavage site HelixII ↓		Helix I	SEQ ID NO:	REFERENCE
R135P target:	aucg	a g**c**c	guacgu	SEQ ID NO:13	Rodriguez *et al.*, 1993
Ribozyme arms:	uagc	aaga	caugca		
P180A target:	acau	c **g**cc	gagggc	SEQ ID NO:14	Daiger *et al.*, 1995
Ribozyme arms:	ugug	aaga	cucccg		
D190G target:	aauc	g **g**cu	acuaca	SEQ ID NO:15	Dryja *et al.*, 1991
Ribozyme arms:	uuag	aaga	ugaugu		
H211R target:	ucgu	g guc	c**g**cuuc	SEQ ID NO:16	Macke *et al.*, 1993
Ribozyme arms:	agcg	aaga	gcgaag		
H211P target:	ucgu	g guc	cccuuc	SEQ ID NO:17	Macke *et al.*, 1993
Ribozyme arms:	agcg	aaga	gggaag		

TABLE 4 (con't)

RIBOZYME	SEQUENCE			SEQ ID NO:	REFERENCE
	Cleavage site				
	HelixII		Helix I		
F220C target:	cauc	u guu	ucugcu	SEQ ID NO:18	Bunge *et al.*, 1993
Ribozyme arms:	guag	aaga	agacga		
P347S target:	aggu	g gcc	ucggcc	SEQ ID NO:19	Dryja *et al.*, 1990
Ribozyme arms:	uccg	aaga	agccgg		

TABLE 5

ILLUSTRATIVE HAMMERHEAD RIBOZYME TARGETS OF THE PRESENT INVENTION

RIBOZYME	SEQUENCE	SEQ ID NO:	REFERENCE
	Target reads 5' to 3'	ribozyme reads 3' to 5'	
ROD OPSIN MRNA-SPECIFIC:			
P23H target:	gcc**a**cuu cgagua	SEQ ID NO:20	Berson *et al.*, 1991
ribozyme arms:	cgguga gcucau		
P23L target:	gcc**u**cuu cgagua	SEQ ID NO:21	Dryja *et al.*, 1991
ribozyme arms:	cggaga gcucau		
Q28H target:	caca**c**ua cuaccu	SEQ ID NO:22	Bunge *et al.*, 1993
ribozyme arms:	guguga gaggga		
F45L target:	aug**g**uuc ugcuga	SEQ ID NO:23	Sung *et al.*, 1991
ribozyme arms:	uaccaa acgacu		
L46R target:	auguuuc **g**gcuga	SEQ ID NO:24	Rodriguez *et al.*, 1993
ribozyme arms:	uacaaa ccgacu		

TABLE 5 (con't)

RIBOZYME	SEQUENCE	SEQ ID NO:	REFERENCE
	Target reads 5' to 3'	ribozyme reads 3' to 5'	
G51R target:	ug**c**gcuu ccccau	SEQ ID NO:25	Dryja *et al.*, 1992
ribozyme arms:	acgcga ggggua		
G51A target:	ugg**c**cuu ccccau	SEQ ID NO:26	Macke *et al.*, 1993
ribozyme arms:	accgga ggggua		
G51V target:	ugg**u**cuu ccccau	SEQ ID NO:27	Dryja *et al.*, 1991
ribozyme arms:	accaga ggggua		
P53R target:	ugggcuu cc**g**cau	SEQ ID NO:28	Inglehearn *et al.*, 1992
ribozyme arms:	acccga ggcgua		
T58R target:	cuuccuc a**g**gcuc	SEQ ID NO:29	Bunge *et al.*, 1993
ribozyme arms:	gaagga uccgag		

TABLE 5 (con't)

RIBOZYME	SEQUENCE	SEQ ID NO:	REFERENCE
	Target reads 5' to 3'	ribozyme reads 3' to 5'	
T58R target:	ca**g**gcuc uacguc	SEQ ID NO:30	Bunge *et al.*, 1993
ribozyme arms:	guccga augcag		
Q64stop target:	caccguc **u**agcac	SEQ ID NO:31	Macke *et al.*, 1993
ribozyme arms:	guggca aucgug		
Q64stop target:	ccguc**u**a gcacaa	SEQ ID NO:32	Macke *et al.*, 1993
ribozyme arms:	ggcaga cguguu		
)68-71 target:	**ug**aacua cauccu	SEQ ID NO:33	Keen *et al.*, 1991
ribozyme arms:	acuuga guagga		
V87D target:	gg**a**ccua gguggc	SEQ ID NO:34	Sung *et al.*, 1991
ribozyme arms:	ccugga ccaccg		

TABLE 5 (con't)

RIBOZYME	SEQUENCE Target reads 5' to 3'	SEQ ID NO: ribozyme reads 3' to 5'	REFERENCE
G90D target:	gug**a**cuu caccag	SEQ ID NO:35	Sieving *et al.*, 1992
ribozyme arms:	cacuga gugguc		
G106W target:	cgucuuc **u**ggccc	SEQ ID NO:36	Sung *et al.*, 1991
ribozyme arms:	gcagaa accggg		
C110Y target:	caggau**a** caauuu	SEQ ID NO:37	Dryja *et al.*, 1992
ribozyme arms:	guccua guuaaa		
G114D target:	agg**a**cuu cuuugc	SEQ ID NO:38	Vaithinathan *et al.*, 1994
ribozyme arms:	uccuga gaaacg		
R135G target:	ag**g**ggua cguggu	SEQ ID NO:39	Bunge *et al.*, 1993
ribozyme arms:	ucccca gcacca		

TABLE 5 (con't)

RIBOZYME	SEQUENCE	SEQ ID NO:	REFERENCE
	Target reads 5' to 3'	ribozyme reads 3' to 5'	
R135L target:	aguggua cguggu	SEQ ID NO:40	Andreasson *et al.*, 1992
ribozyme arms:	ucacca gcacca		
R135L target:	aguugua cguggu	SEQ ID NO:41	Jacobson *et al.*, 1991
ribozyme arms:	ucaaca gcacca		
R135P target:	agccgua cguggu	SEQ ID NO:42	Rodriguez *et al.*, 1993
ribozyme arms:	ucggca gcacca		
C140S target:	uggugu**c** uaagcc	SEQ ID NO:43	Macke *et al.*, 1993
ribozyme arms:	accaca auucgg		
P171L target:	accc**cua** cucgcc	SEQ ID NO:44	Dryja *et al.*, 1991
ribozyme arms:	uggga gagcgg		

TABLE 5 (con't)

RIBOZYME	SEQUENCE Target reads 5' to 3'	SEQ ID NO: ribozyme reads 3' to 5'	REFERENCE
P171L target:	cc**u**acuc gccggc	SEQ ID NO:45	Dryja *et al.*, 1991
ribozyme arms:	ggauga cggccg		
P171S target:	cacccuc acucgc	SEQ ID NO:46	Stone *et al.*, 1993
ribozyme arms:	guggga ugagcg		
Y178C target:	gu**g**cauc cccgag	SEQ ID NO:47	Farrar *et al.*, 1991
ribozyme arms:	cacgua gggcuc		
P180A target:	guacauc **g**ccgag	SEQ ID NO:48	Daiger *et al.*, 1995
ribozyme arms:	caugua cggcuc		
C187Y target:	gcucgu**a** uggaau	SEQ ID NO:49	Nathans *et al.*, 1993
ribozyme arms:	cgagca accuua		

TABLE 5 (con't)

RIBOZYME	SEQUENCE	SEQ ID NO:	REFERENCE
	Target reads 5' to 3'	ribozyme reads 3' to 5'	
G188R target:	ucgugua gaaucg	SEQ ID NO:50	Dryja *et al.*, 1991
ribozyme arms:	agcaca cuuagc		
D190G target	uggaauc ggcuac	SEQ ID NO:51	Dryja et al., 1991
ribozyme arms:	accuua ccgaug		
D190Y target:	gaaucua cuacua	SEQ ID NO:52	Fishman *et al.*, 1992
ribozyme arms:	cuuaga gaugau		
M207R target:	cagguuc gugguc	SEQ ID NO:53	Farrar *et al.*, 1992
ribozyme arms:	guccaa caccag		
H211R target:	cgugguc cgcuuc	SEQ ID NO:54	Macke *et al.*, 1993
ribozyme arms:	gcacca gcgaag		

TABLE 5 (con't)

RIBOZYME	SEQUENCE Target reads 5' to 3'	SEQ ID NO: ribozyme reads 3' to 5'	REFERENCE
H211P target:	cgugguc cccuuc	SEQ ID NO:55	Macke *et al.*, 1993
ribozyme arms:	gcacca gggaag		
C264X target:	ccugaauc **ugggug**	SEQ ID NO:56	Vaithinathan *et al.*, 1993
ribozyme arms:	ggacuua acccac		
P267L target:	ggugcuc uacgcc	SEQ ID NO:57	Fishman *et al.*, 1992
ribozyme arms:	ccacga augcgg		
F220C target:	uaucauc uguuuc	SEQ ID NO:58	Bunge *et al.*, 1993
ribozyme arms:	auagua acaaag		
F220C target:	cuguuuc ugcuau	SEQ ID NO:59	Bunge *et al.*, 1993
ribozyme arms:	gacaaa acgaua		

TABLE 5 (con't)

RIBOZYME	SEQUENCE Target reads 5' to 3'	SEQ ID NO: ribozyme reads 3' to 5'	REFERENCE
C222R target:	ucuuuuc cgcuau	SEQ ID NO:60	Bunge *et al.*, 1993
ribozyme arms:	agacaa gcgaua		
A292E target:	agaguuc uuugcc	SEQ ID NO:61	Dryja *et al.*, 1993
ribozyme arms:	ucucaa aaacgg		
Q344stop target:	cgagcua gguggc	SEQ ID NO:62	Sung *et al.*, 1991
ribozyme arms:	gcucga ccaccu		
P347S target:	uggccuc ggccua	SEQ ID NO:63	Dryja *et al.*, 1990
ribozyme arms:	accgga ccggau		
RP1 mRNA-SPECIFIC:			
R677stop target:	aaaaaauc uugaca	SEQ ID NO:64	Pierce *et al.*, 1999
ribozyme arms:	uuuuuua aacugu		

Table 5 (con't)

Ribozyme	Sequence	SEQ ID NO:	Reference
	Target reads 5' to 3'	ribozyme reads 3' to 5'	
RDS/Peripherin mRNA-Specific:			
C118 target:	ggcucuc ugcuuuc	SEQ ID NO:65	Farrar *et al.*, 1991
ribozyme arms:	ccgaga acgaaag		
R172Q target:	gguuuuc aggacu	SEQ ID NO:66	Wells *et al.*, 1993
ribozyme arms:	ccaaaa uccuga		
R172W target:	gguuuuu gggacu	SEQ ID NO:67	Wells *et al.*, 1993
ribozyme arms:	ccaaaa cccuga		
P210R target:	guccguu ucagcu	SEQ ID NO:68	Jackson *et al.*, 1993
ribozyme arms:	caggca agucga		
C214S target:	gcugcuc caaucc	SEQ ID NO:69	Keen and Inglehearn, 1996
ribozyme arms:	cgacga guuagg		

TABLE 5 (con't)

RIBOZYME	SEQUENCE	SEQ ID NO:	REFERENCE
	Target reads 5' to 3'	ribozyme reads 3' to 5'	
P216L target:	aaucuua gcucgc	SEQ ID NO:70	Kajiwara *et al.*, 1991
ribozyme arms:	uuagaa cgagca		
P219 target:	cuagcuc gc**ggcc**	SEQ ID NO:71	Kajiwara *et al.*, 1991
ribozyme arms:	gaucga cgccgg		

TABLE 6

ADDITIONAL ILLUSTRATIVE HAIRPIN RIBOZYME TARGETS OF THE PRESENT INVENTION

RIBOZYME	SEQUENCE			SEQ ID NO:	REFERENCE
	Cleavage site				
	HelixII	↓	Helix I		
RDS/PERIPHERIN MRNA-SPECIFIC:					
C118 target:	ucuc	u gcu	**uucugc**	SEQ ID NO:72	Farrar *et al.*, 1991
Ribozyme arms:	agag	aaga	aagacg		
R172W target:	caac	g guu	uuuggg	SEQ ID NO:73	Wells *et al.*, 1993
Ribozyme arms:	guug	aaga	aaaccc		
P210R target:	cguc	c **g**uu	ucagcu	SEQ ID NO:74	Jackson *et al.*, 1993
Ribozyme arms:	gcag	aaga	agucga		
C214S target:	cagc	u gcu	**c**caauc	SEQ ID NO:75	Keen and Inglehearn 1996
Ribozyme arms:	gucg	aaga	gguuag		

TABLE 6 (con't)

RIBOZYME	SEQUENCE	SEQ ID NO:	REFERENCE
	<u>Cleavage site</u> HelixII ↓ Helix I		
P216L target:	ucuu a gcu cgccac	SEQ ID NO:76	Kajiwara *et al.*, 1991
Ribozyme arms:	agag aaga gcggug		
P219 target:	uccu a gcu cgc**ggc**	SEQ ID NO:77	Kajiwara *et al.*, 1991
Ribozyme arms:	aggg aaga gcgccg		

In copending application serial number 09/063,667, the inventors demonstrated that AAV-vectored ribozymes could be used as a therapy for a variety of retinal diseases, including, for example, diseases caused by the presence of mutant forms of rod opsin polypeptide-spefici mRNA. Through the use of selected ribozymes, it was demonstrated that mRNAs encoding these mutated rod opsin polypeptides could be selectively cleaved, and thus, inactivated by such AAV-vectored ribozyme compositions. In similar fashion, a series of ribozymes have been constructed and tested that are relevant to the treatment of diabetic retinopathy, a leading cause of blindness.

The chief characteristic of diabetic retinopathy is retinal neovascularization-the pathologic spread of blood vessels in the eye. Unique ribozymes have been developed which target the mRNAs that encode various proteins involved in this process. These include ribozymes directed at the wild-type mRNA for the adenosine A2b receptor, for IGF-I (insulin-like growth factor-1) receptor, for inducible nitric oxide synthase (iNOS), and for several integrins implicated in retinal neovascularization (*e.g.*, alpha1, alpha3, alphaV). The sequences of these ribozymes and their kinetic characterization are shown in Table 7. Also shown in Table 7 are the analyses of P347S ribozymes, that are specific for another mutant form of the rod opsin polypeptide. The nucleotide sequence of each of these exemplary ribozymes is presented in Table 8.

Insulin-like growth factor-I accounts for much of the growth-stimulating properties of serum and activates cells to proceed through the cell cycle. IGF-I receptors are present in the microvascular cells of the retina, and IGF-I can induce angiogenesis (proliferation of blood vessels) in the retina in response to VEGF exposure. The migration of endothelial cells is dependent on alpha1, alpha3 and alphaV integrins, which promote cell-cell contact. Adenosine also promotes angiogenesis. Adenosine is a mediator of changes in blood flow in response to oxygen deprivation (ischemia), which may serve as the ultimate stimulus for retinal neovascularization. There are a variety of adenosine receptors in the retina that control both vasodialtion and angiogenesis. The inventors hypothesize that the A2b receptor is involved in controlling the proliferation of new blood vessels. Finally, an increase in NO (nitric oxide) appears to stimulate a disruption of the blood-retinal barrier, and this increase correlates with an increase of inducible nitric oxide synthase (iNOS or NOS2). Reducing expression of this form of nitric oxide synthase appears to prevent retinal neovascularization by maintaining the normal blood-retinal barrier.

TABLE 7

KINETIC ANALYSES OF EXEMPLARY RIBOZYME CONSTRUCTS OF THE PRESENT INVENTION

Experiment #	Ribozyme	Vmax (nM/min)	Km (nM)	kcat (min-1)	Target Sequence
3382	P347S pig cloned	7.8	1645.9	1.3	AGGCGUCAGCCUA
3386	P347S pig cloned	6.7	1680.0	1.1	(SEQ ID NO:78
3879 A	P347S pig	15.8	2836.0	1.1	
3879 B	P347S pig	17.7	977.0	1.2	
3901 A	P347S pig	27.3	141618.0	1.8	
3901 B	P347S pig	31.9	181703.0	2.2	
	average	7.3	55076.7	1.5	
	std dev	0.5	553.2	0.4	
Anna1	P347S human	0.00200	20325.0	0.000163	UGGCCUCGGCCUA
Anna2	P347S human	0.00070	63424.0	0.000056	(SEQ ID NO:79
	average	1.35E-03	4.19E+04	1.10E-04	
	std dev	5.31E-04	1.76E+04	4.37E-05	
3837 A	A2B Rz1	666.7	5070.3	44.4	CAUGUCUCUUUG
3837 B	A2B Rz1	416.7	3628.6	27.8	(SEQ ID NO:80)
	average	541.7	4349.5	36.1	
	std dev	125.0	720.9	8.3	
3856 A	iNOS	1.8	491.4	0.12	GGCCUGUCCUUGGA
3856 B	iNOS	1.9	392.8	0.13	(SEQ ID NO:81)
	average	1.9	442.1	0.13	
	std dev	0.1	49.3	0.005	
3872 A	alpha 1 Rz1	61.7	15550	4.1	AGAUGUCUAUAAG
3872 B	alpha 1 Rz1	52.4	35688	3.5	(SEQ ID NO:82)
	average	57.0	25619	3.8	
	std dev	4.7	10069	0.3	
3873 A	alpha 1 Rz2	31.0	30606.0	2.1	GAGAGUCUCAUGA
3873 B	alpha 1 Rz2	53.5	53775.0	3.6	(SEQ ID NO:83)
	average	42.2	42190.5	2.9	
	std dev	11.3	11584.5	0.8	
3885 A	alpha V Rz1	30.5	4982.0	2.0	GCGCGUCUUCCCG
3885 B	alpha V Rz1	36.2	5410.0	2.4	(SEQ ID NO:84)
	average	33.4	5196.0	2.2	
	std dev	2.9	214.0	0.2	
3886 A	alpha V Rz2	238.1	8537.0	15.9	
3886 B	alpha V Rz2	333.3	14043.0	22.2	ACUGGUCUUCUAC
	average	285.7	11290.0	19.1	(SEQ ID NO:85)
	std dev	47.6	2753.0	3.1	

TABLE 7 (con't)

Experiment #	Ribozyme	Vmax (nM/min)	Km (nM)	kcat (min-1)	Target Sequence
3840 A	alpha 3 Rz 1	5.4	3620.0	0.4	CUAUGUCUUCAUG
3840 B	alpha 3 Rz 1	6.9	4693.0	0.5	(SEQ ID NO:86)
	average	6.2	4156.5	0.4	
	std dev	1.1	758.7	0.1	
3841 A	alpha 3 Rz 2	2.8	1655.0	0.2	CGCUGUCUUCCAC
3841 B	alpha 3 Rz 2	2.2	1240.0	0.2	(SEQ ID NO:87)
	average	2.5	1447.5	0.2	
	std dev	0.424	293.449	0.028	
3859	alpha 5 Rz 2	322.6	81112.0	21.5	UCCUGUCUGCCAC (SEQ ID NO:106)
3925 A*	IGF1 Rz 1	7.2	48301.0	0.48	CUUCGUCUUUGCA
3925 B*	IGF1 Rz 1	6.8	45830.0	0.46	(SEQ ID NO:88)
	average	7.0	47065.5	0.47	
	std dev	0.3	1747.3	0.01	
3905	IGF1 Rz 2	27.6	9757.0	18.40	GUACGUCUUCCAU
3924 A*	IGF1 Rz 2	3.3	1902.0	0.22	(SEQ ID NO:89)
3924 B*		2.4	1706.0	0.16	
	average	2.8	1804.0	0.2	
	std dev	0.60	138.59	0.04	
4097	VEGFR1 Rz1 mouse	344.8	6877.6	23.0	GGGUGUCUAUAGG (SEQ ID NO:107)
	VEGFR1 Rz1 mouse				ACAAGUCAAACCU (SEQ ID NO:108)
	VEGFR2 Rz1 mouse				ACUUGUCUUAAAU (SEQ ID NO:109)
	VEGFR2 Rz1 mouse				CAUGGUCUUCUGU (SEQ ID NO:110)

TABLE 8

SEQUENCE OF EXEMPLARY RIBOZYME CONSTRUCTS OF THE PRESENT INVENTION

Ribozyme	Sequence
A2B Rz1	CAAAGACUGAUGAGCCGUUCGCGGCGAAACAUGU (SEQ ID NO:90)
A2B Rz 2	GGCAUACUGAUGAGCCGUUCGCGGCGAAACAAUG (SEQ ID NO:91)
ALPHA 3 RZ 1	CAUGAACUGAUGAGCCGUUCGCGGCGAAACAUAG (SEQ ID NO:92)
ALPHA 3 RZ 2	GUGGAACUGAUGAGCCGUUCGCGGCGAAACAGCG (SEQ ID NO:93)
ALPHA 5 Rz 1	GAGGUACUGACGAGCCGUUCGCGGCGAAACAGCA (SEQ ID NO:94)
ALPHA 5 Rz 2	GUGGCACUGAUGAGCCGUUCGCGGCGAAACAGGA (SEQ ID NO:95)
ALPHA 1 Rz 1	CUUAUACUGAUGAGCCGUUCGCGGCGAAACAUCU (SEQ ID NO:96)
ALPHA 1 Rz 2	UCAUGACUGAUGAGCCGUUCGCGGCGAAACUCUC (SEQ ID NO:97)
ALPHA V Rz 1	CGGGAACUGAUGAGCCGUUCGCGGCGAAACGCGC (SEQ ID NO:98)
ALPHA V Rz 2	GUAGAACUGAUGAGCCGUUCGCGGCGAAACCAGU (SEQ ID NO:99)

TABLE 8 (con't)

Ribozyme	Sequence
IGF1 Rz 1	UGCAAACUGAUGAGCCGUUCGCGGCGAAACGAAG (SEQ ID NO:100)
IGF1 Rz 2	GGAACUGAUGAGCCGUUCGCGGCGAAACGUAC (SEQ ID NO:101)
P347S pig	UAGGCUCUGAUGAGCCGCUUCGGCGGCAAACGCCU (SEQ ID NO:102)
P347S human	UAGGCCCUGAUGAGCCGCUUCGGCGGCAAAGGCCA (SEQ ID NO:103)
iNOS	GCCCCAAGCUGAUGAGCCGCUUCGGCGGCGAAACAGG (SEQ ID NO:104)
VEFGR1 Rz1	CCUAUACUGAUGAGCCGUUCGCGGCGAAACACCC (SEQ ID NO:111)
VEFGR1 Rz2	AGGUUUCUGAUGAGCCGUUCGCGGCGAAACUUGU (SEQ ID NO:112)
VEFGR2 Rz1	ACAGAACUGAUGAGCCGUUCGCGGCGAAACCAUG (SEQ ID NO:113)
VEFGR2 Rz2	AUUUAACUGAUGAGCCGUUCGCGGCGAAACAAGU (SEQ ID NO:114)

5.6 EXAMPLE 6 – THE ROP MOUSE MODEL FOR DIABETIC RETINOPATHY

Diabetic retinopathy is characterized by the formation of new blood vessels on the surface of the retina. This neovascularization results in damage to the retina and eventual blindness. A model for diabetic retinopathy called the ROP mouse (ROP stands for retinopathy of pre-maturity) has been developed in which, under the proper conditions, neovascularization on the surface of the retina can be stimulated. These conditions are:

On postnatal day 7 the mouse pups are placed into a high oxygen (75%) environment. Then, on postnatal day 12 the mouse pups are returned to a normal oxygen environment. This lowering of the oxygen simulates a state of hypoxia within the retina of mouse pups. It is this hypoxia that stimulates the onset of neovascularization on the surface of the retina. On postnatal day 17 the mouse pups are sacrificed and their whole eyes are taken and sectioned for analysis. Using these sections, the cross sections of the new blood vessels that have formed on the surface of the retina can be readily observed. With proper staining the nuclei of the endothelial cells that make-up these new blood vessels can also be readily counted. The extent of neovascularization is determined by the number of nuclei that can be counted on the surface of the retina per section of eye.

This ROP mouse represents an accurate and facile model system that can be employed to test the effect of particular AAV-vectored ribozyme constructs on the formation of neovascularization on the surface of the mammalian retina.

5.7 EXAMPLE 7 – THE INSULIN-LIKE GROWTH FACTOR 1 RECEPTOR RIBOZYME

A hammerhead ribozyme has been designed and tested *in vitro* and *in vivo* that targets and cleaves the mRNA for the insulin-like growth factor receptor (IGF-1R). This ribozyme has been designated IGF1R Rz1 (SEQ ID NO:105). This ribozyme was designed and tested, *in vitro*, prior to cloning the gene encoding this ribozyme into the pTRUP21 adeno associated

virus (AAV) vector. Once cloned into the AAV vector, the AAV-IGF1R Rz1 construct was injected into the eyes of the ROP mouse model.

In an exemplary study, this AAV-IGF1R Rz1 construct was injected into the vitreous of the right eye the mouse pups on postnatal day 1. The left eye received no injection and served as a control. A total of 10 mice were used in this study. On postnatal day 17 the mice were sacrificed and the eyes were sectioned. The number of nuclei in endothelial cells found above the surface of the retina was counted in three sections for each eye (injected and uninjected). On average the uninjected eyes had 55 neovascular nuclei per section while the AAV-IGF1R Rz injected eyes had an average of 27 neovascular nuclei per section (FIG. 10). Statistical analyses confirmed that this finding was a "very highly significant result" with a $p = 3.56 \times 10^{-7}$. The results of this study confirmed that AAV-vectored ribozyme constructs, such as IGF1R Rz1, can inhibit the formation of neovascularization on the surface of the mouse retina.

5.8 EXAMPLE 8 – COMPOSITIONS USING P21NEWHP AAV PACKAGING VECTORS

Two ribozymes for the IGF-1 receptor (IGF-1R) and one for the Adenosine A_{2b} receptor were cloned into the p21NewHp AAV packaging vector. These plasmids have been used to transfect human retinal endothelial cells (HREC) isolated from human donor retinas. These plasmids have also been tested in the mouse model of oxygen-induced retinopathy of prematurity (ROP). Below are the results of these studies.

5.8.1 EFFECT OF IGF-1R RIBOZYMES ON MIGRATION OF HRECS

Active and inactive IGF-1R ribozymes and the cloning vector p21NewHp (FIG. 13) were transfected into HRECs (human retinal endothelial cells) to determine their effect on the ability of the cells to migrated in a modified Boyden chamber (Glaser *et al.*, 1980). Cells transfected with plasmid coding for active IGF-1R Rz1 reduced migration of cells by an average of 91% when compared to the p21NewHp control at all concentrations of IGF1 (1, 10 and 100 ng/ml), and cells transfected with plasmid coding for the inactive IGF-1R (Rz1i) reduced migration of cells by an average of 60% (FIG. 15A). Cells transfected with plasmid coding for active IGF-1R Rz2 reduced migration of cells by an average of 45% when compared to controls (FIG. 15B). These results demonstrate that these ribozymes are effective at reducing the function of the IGF-1R protein in cell culture and that a strong antisense effect can result from transfection with the inactive version of the ribozymes.

5.8.2 EFFECT OF IGF-1R RIBOZYMES ON NEOVASCULARIZATION IN THE ROP MOUSE MODEL

Active and inactive IGF-1R ribozymes and the cloning vector p21NewHp were injected intraocularly on post-natal day one in the right eye of mouse pups, with no injection in the left eye, and the pups and their dams were taken through the oxygen-induced model of ROP. The extent of angiogenesis was determined and is shown in FIG. 14. The cloning vector p21NewHp had no effect on angiogenesis. The active IGF-1R Rz1 and the active IGF-1R Rz2 reduced the average number of nuclei per section on average by 65% and 52%, respectively. The inactive IGF-1R Rz1 reduced the average number of nuclei per section on average by 17% while the inactive IGF-1R Rz2 had no significant effect. These results demonstrate that the active versions of the ribozymes are functional *in vivo* as demonstrated by the significant reduction in the formation of neovascularization on the retinas of the ROP mice. In addition, this data show that the strong antisense effect for the inactive version of the IGF-1R Rz1 is greatly reduced *in vivo* and that there is very little or no antisense effect found with the inactive version of the IGF-1R Rz2.

5.8.3 EFFECT OF A_{2B} RIBOZYME ON NEOVASCULARIZATION IN THE ROP MOUSE MODEL

The active and inactive A_{2b} ribozyme and the cloning vector p21NewHp were injected intraocularly on post-natal day one in the right eye of mouse pups, with no injection in the left eye, and the pups and their dams were taken through the oxygen-induced model of ROP. The extent of angiogenesis was determined and is shown in FIG. 16. The cloning vector p21NewHp had no effect on angiogenesis. The active A_{2b} Rz2 reduced the average number of nuclei per section on average by 54% while the inactive A_{2b} Rz2 shows no significant antisense effect.

6. REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference in whole or in part:

U. S. Patent 4,683,195.

U. S. Patent 4,683,202.

U. S. Patent 4,800,159.

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All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

CLAIMS:

1. A composition comprising:

(a) at least a first ribozyme that specifically cleaves an mRNA encoding a protein, polypeptide, or peptide selected from the group consisting of rod opsin, RDS/peripherin, iNOS, VEGFR1, VEGFR2, Adenosine A_{2B} receptor, IGF-1, integrin alpha 1, integrin alpha 3, integrin alpha 5, and integrin alpha V; wherein said ribozyme: (i) comprises at least a first nucleotide sequence selected from any one of SEQ ID NO:2, SEQ ID NO:90 to SEQ ID NO:104, or SEQ ID NO:111 to SEQ ID NO:114; or (ii) specifically cleaves an mRNA comprising at least a first target sequence selected from any one of SEQ ID NO:1, SEQ ID NO:3 to SEQ ID NO:89, and SEQ ID NO:106 to SEQ ID NO:110;

(b) a vector comprising a polynucleotide encoding said ribozyme, said polynucleotide operably positioned downstream of at least a first promoter that directs expression of said polynucleotide in a selected mammalian cell transformed with said vector;

(c) a viral particle comprising said ribozyme or said polynucleotide;

(d) an adeno-associated viral vector comprising said ribozyme or said polynucleotide; or

(e) a host cell comprising said ribozyme or said polynucleotide.

2. The composition according to claim 1, wherein said ribozyme specifically cleaves an mRNA comprising at least a first target sequence selected from any one of SEQ ID NO:1, SEQ ID NO:3 to SEQ ID NO:89, and SEQ ID NO:106 to SEQ ID NO:110.

3. The composition according to claim 2, wherein said ribozyme specifically cleaves an mRNA comprising at least a first target sequence selected from any one of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, SEQ ID NO:62, SEQ ID NO:63, SEQ ID NO:64, SEQ ID NO:65, SEQ ID NO:66, SEQ ID NO:67, SEQ ID NO:68, SEQ ID NO:69, SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO:73, SEQ ID NO:74, SEQ ID NO:75, SEQ ID NO:76, SEQ ID NO:77, SEQ ID NO:78, SEQ ID NO:79, SEQ ID NO:80, SEQ ID NO:81, SEQ ID NO:82, SEQ ID NO:83, SEQ ID NO:84, SEQ ID NO:85, SEQ ID NO:86, SEQ ID NO:87, SEQ ID NO:88, SEQ ID NO:89, SEQ ID NO:106, SEQ ID NO:107, SEQ ID NO:108, SEQ ID NO:109, and SEQ ID NO:110.

4. The composition according to claim 1, wherein said ribozyme comprises at least a first nucleotide sequence selected from any one of SEQ ID NO:2, SEQ ID NO:90 to SEQ ID NO:104, or SEQ ID NO:111 to SEQ ID NO:114.

5. The composition according to claim 4, wherein said ribozyme comprises at least a first sequence selected from the group consisting of SEQ ID NO:90, SEQ ID NO:91, SEQ ID NO:92, SEQ ID NO:93, SEQ ID NO:94, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:100, SEQ ID NO:101, SEQ ID NO:102, SEQ ID NO:103, SEQ ID NO:104, SEQ ID NO:111, SEQ ID NO:112, SEQ ID NO:113, and SEQ ID NO:114.

6. The composition according to claim 1, wherein said ribozyme specifically cleaves an mRNA encoding a protein, polypeptide, or peptide selected from the group consisting of rod opsin, RP1, RDS/peripherin, iNOS, Adenosine A_{2B} receptor, IGF-1, IGF-1 receptor, integrin alpha 1, integrin alpha 5, and integrin alpha V, wherein said protein, polypeptide or peptide comprises at least a first mutation in its primary amino acid sequence.

7. The composition according to claim 6, wherein said ribozyme specifically cleaves an mRNA encoding a protein, polypeptide, or peptide selected from the group consisting of rod opsin, RP1, RDS/peripherin, iNOS, Adenosine A_{2B} receptor, IGF-1, IGF-1 receptor, integrin alpha 1, integrin alpha 5, and integrin alpha V, wherein said protein, polypeptide or peptide further comprises at least a second mutation in its primary amino acid sequence.

8. The composition according to claim 7, wherein said ribozyme specifically cleaves an mRNA encoding a protein, polypeptide, or peptide selected from the group consisting of rod opsin, RP1, RDS/peripherin, iNOS, Adenosine A_{2B} receptor, IGF-1, IGF-1 receptor, integrin alpha 1, integrin alpha 5, and integrin alpha V, wherein said protein, polypeptide or peptide further comprises at least a third mutation in its primary amino acid sequence.

9. The composition according to claim 1, wherein said ribozyme specifically cleaves an mRNA encoding a mutant polypeptide more effectively than said ribozyme specifically cleaves an mRNA encoding the corresponding wild-type polypeptide.

10. The composition according to claim 1, wherein said ribozyme specifically cleaves an mRNA encoding a mutant polypeptide, but does not cleave an mRNA encoding the corresponding wild-type polypeptide.

11. The composition according to claim 1, wherein said ribozyme is a hairpin ribozyme.

12. The composition according to claim 1, wherein said ribozyme is a hammerhead ribozyme.

13. The composition according to claim 1, wherein said vector is a viral vector.

14. The composition according to claim 13, wherein said viral vector is an adeno-associated viral vector.

15. The composition according to claim 14, wherein said viral vector is an adeno-associated viral serotype 1 (AAV1), serotype 2 (AAV2), serotype 3 (AAV3), serotype 4 (AAV4), serotype 5 (AAV5), or serotype 6 (AAV6) vector.

16. The composition according to claim 1, wherein said at least a first promoter directs expression of said polynucleotide in a retinal cell.

17. The composition according to claim 16, wherein said at least a first promoter directs expression of said polynucleotide in a retinal photoreceptor cell.

18. The composition according to claim 16, wherein said at least a first promoter directs expression of said polynucleotide in a retinal photoreceptor rod or cone cell.

19. The composition according to claim 16, wherein said at least a first promoter directs expression of said polynucleotide in a Mueller cell, or a retinal pigement epithelium cell.

20. The composition according to claim 16, wherein said at least a first promoter comprises a constitutive, inducible, tissue-specific, or cell-specific promoter.

21. The composition according to claim 20, wherein said at least a first promoter comprises a retinal-specific or an eye-specific promoter.

22. The composition according to claim 16, wherein said promoter is selected from the group consisting of a CMV promoter, a β-actin promoter, a rod opsin promoter, a hybrid CMV promoter, a hybrid β-actin promoter, an EF1 promoter, a U1a promoter, a U1b promoter, a Tet-inducible promoter and a VP16-LexA promoter

23. The composition according to claim 22, wherein said promoter is a chicken β-actin promoter.

24. The composition according to claim 1, wherein said viral particle is an adenovirus or an adeno-associated viral particle.

25. The composition according to claim 1, wherein said host cell is a mammalian host cell.

26. The composition according to claim 25, wherein said mammalian cell is a human cell.

27. The composition according to claim 26, wherein said human cell is a retinal cell.

28. The composition according to claim 1, further comprising at least a first pharmaceutical excipient.

29. The composition according to claim 28, wherein said at least a first pharmaceutical excipient is suitable for ocular or subretinal administration to a mammalian eye.

30. The composition according to claim 1, further comprising a lipid, a liposome, a nanoparticle, or a microsphere.

31. The composition according to claim 1, further comprising at least a first ocular therapeutic drug.

32. A composition according to any preceding claim, for use in treating a disease or dysfunction of the mammalian eye.

33. The composition according to claim 32, for use in treating a retinal disease or retinal dysfunction.

34. The composition according to claim 33, for use in treating retinopathy.

35. The composition according to claim 34, for use in treating diabetic retinopathy.

36. The composition according to claim 33, for use in treating macular degeneration.

37. The composition according to claim 36, for use in treating age-related macular degeneration.

38. Use of a composition according to any one of claims 1 to 37, in the manufacture of a medicament for treating a disease or dysfunction of the mammalian eye.

39. Use according to claim 38, in the manufacture of a medicament for treating a disease or dysfunction of the human eye.

40. Use according to claim 38 or claim 39, wherein said disease is autosomal dominant retinitis.

41. Use according to any one of claims 38 to 40, wherein said disease is diabetic retinopathy.

42. Use according to any one of claims 38 to 41, wherein said dysfunction is blood-retinal barrier dysfunction.

43. A method for decreasing the amount of mRNA encoding a selected polypeptide in a retinal cell of a mammalian eye, comprising providing to said eye an amount of the composition according to any one of claims 1 to 37, and for a time effective to specifically cleave said mRNA in said cell, and thereby decrease the amount of mRNA in said cell.

44. The method according to claim 43, wherein said ribozyme specifically cleaves an mRNA encoding a polypeptide that causes a pathological condition in, or contributes to a disease, disorder, or dysfunction in a cell or a tissue of a mammalian eye.

45. The method according to claim 44, wherein said ribozyme specifically cleaves an mRNA encoding a polypeptide that causes a pathological condition in, or contributes to a disease, disorder, or dysfunction in a cell or a tissue of a human eye.

46. The method according to any one of claims 43 to 45, wherein said composition is provided to said eye by systemic or direct administration.

47. The method according to any one of claims 43 to 46, wherein said composition is provided to said eye by ocular injection, intravitreolar injection, retinal injection, or subretinal injection.

48. The method according to any one of claims 43 to 47, wherein said pathological condition is selected from the group consisting of retinal degeneration, autosomal dominant retinitis pigmentosa, autosomal recessive retinitis pigmentosa, macular degeneration, age-related macular degeneration, retinopathy, and diabetic retinopathy.

49. The method according to any one of claims 43 to 48, wherein said pathological condition is selected from the group consisting of retinal degeneration, autosomal dominant retinitis pigmentosa, age-related macular degeneration, and diabetic retinopathy.

50. A method for decreasing the amount of a selected polypeptide in a cell or tissue of a mammalian eye, comprising providing to said eye an amount of the composition according to any one of claims 1 to 37, and for a time effective to specifically decrease the amount of said selected polypeptide in said cell or said tissue.

51. A method for decreasing the amount of a selected polypeptide in the eye of a mammal suspected of having a pathological condition selected from the group consisting of retinal degeneration, retinitis, macular degeneration, and retinopathy, comprising directly administering to said eye the composition according to any one of claims 1 to 37, in an amount and for a time effective to specifically cleave an mRNA encoding said selected polypeptide, and thereby decreasing the amount of said polypeptide in said eye.

52. A method for treating, decreasing the severity, or ameliorating the symptoms of a pathological condition that results from the expression of at least a first selected polypeptide in a cell or a tissue of a human eye, said method comprising directly administering to said eye the composition according to any one of claims 1 to 37, in an amount and for a time effective to treat, decrease the severity, or ameliorate the symptoms of said pathological condition.

53. The method according to claim 52, wherein said symptoms are selected from the group consisting of atrophic lesions of the eye, pigmented lesions of the eye, blindness, a reduction in central vision, a reduction in peripheral vision, and a reduction in total vision.

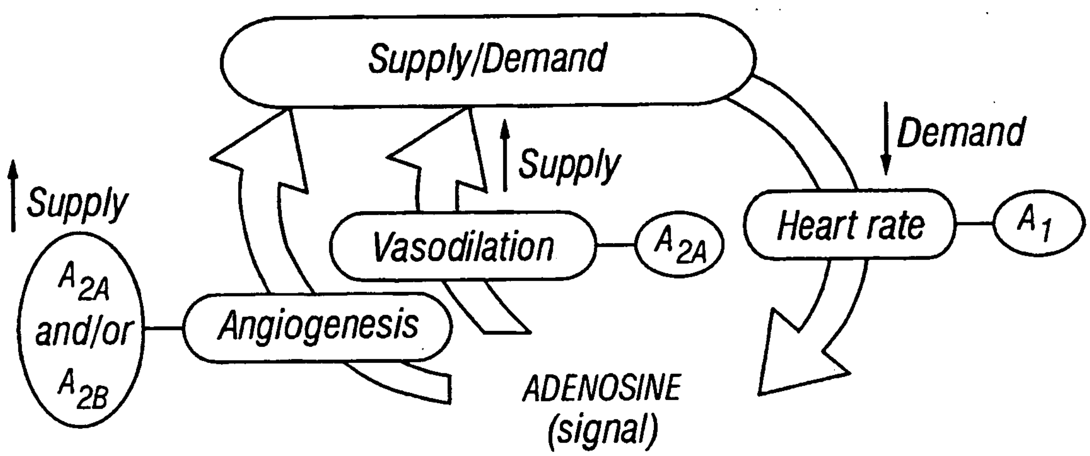

Supply/Demand
↑ Supply
↓ Demand
↑ Supply
Vasodilation
A_{2A}
Heart rate
A_1
A_{2A} and/or A_{2B}
Angiogenesis
ADENOSINE (signal)

FIG. 1

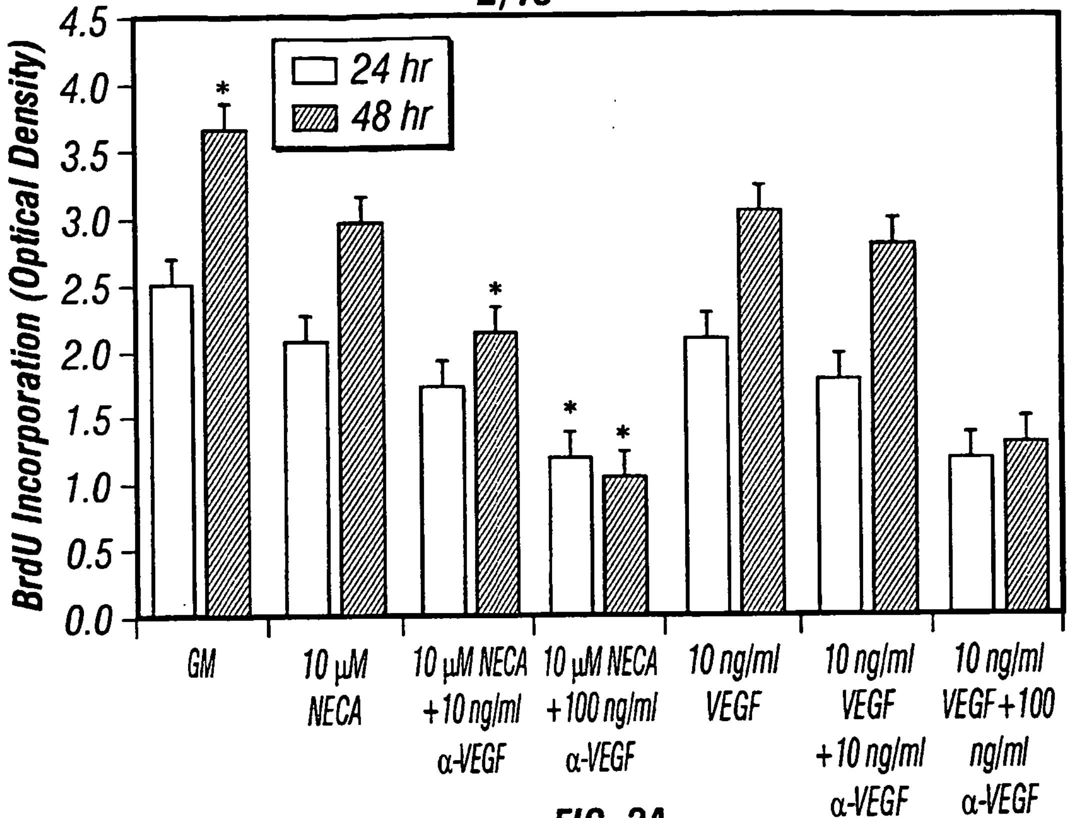

BrdU Incorporation (Optical Density)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
24 hr
48 hr
*
GM
10 μM NECA
10 μM NECA +10 ng/ml α-VEGF
10 μM NECA +100 ng/ml α-VEGF
10 ng/ml VEGF
10 ng/ml VEGF +10 ng/ml α-VEGF
10 ng/ml VEGF+100 ng/ml α-VEGF

FIG. 2A

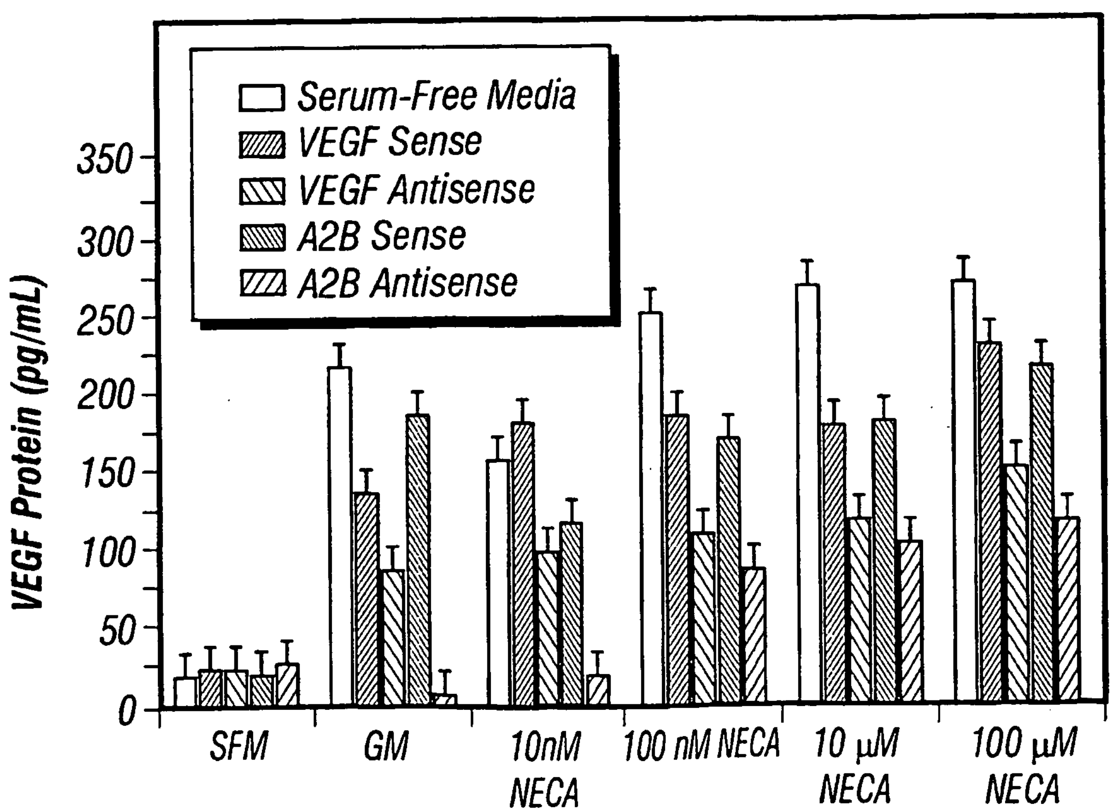

VEGF Protein (pg/mL)
350
300
250
200
150
100
50
0
Serum-Free Media
VEGF Sense
VEGF Antisense
A2B Sense
A2B Antisense
SFM
GM
10nM NECA
100 nM NECA
10 μM NECA
100 μM NECA

FIG. 2B

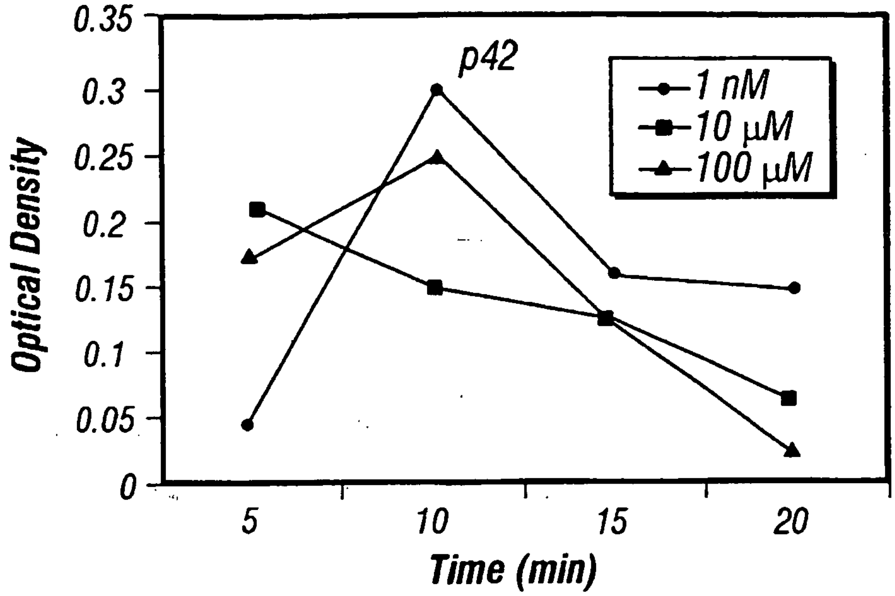

p42
1 nM
10 μM
100 μM
Optical Density
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
5
10
15
20
Time (min)

FIG. 3A

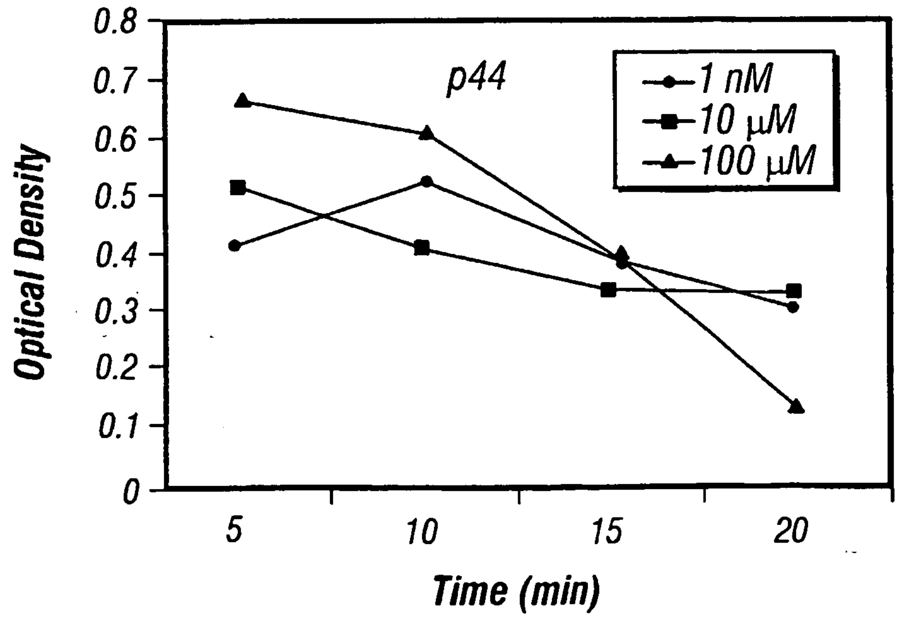

p44
1 nM
10 μM
100 μM
Optical Density
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
5
10
15
20
Time (min)

FIG. 3B

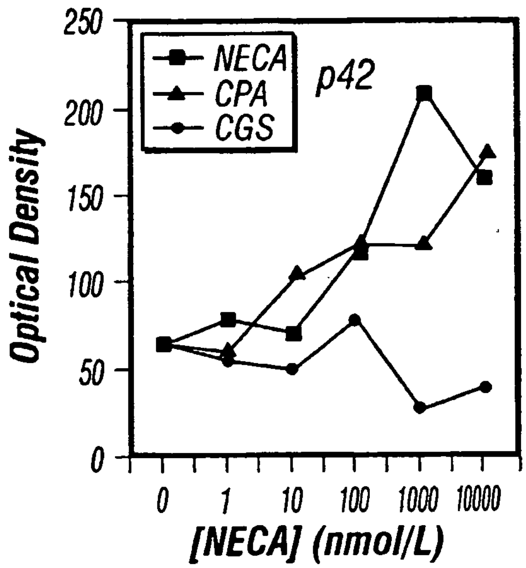


FIG. 4A

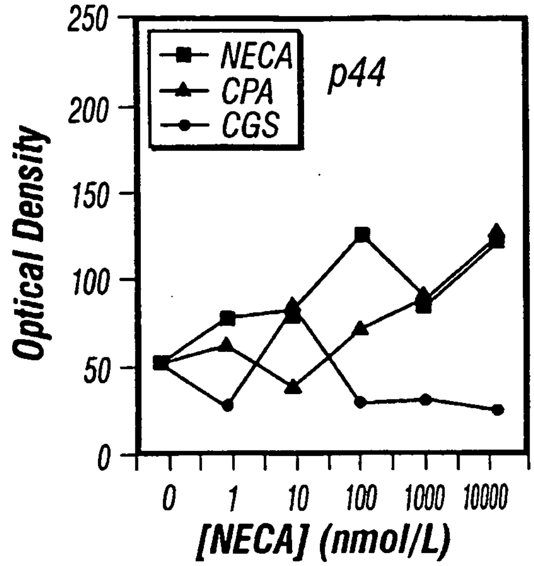


FIG. 4B

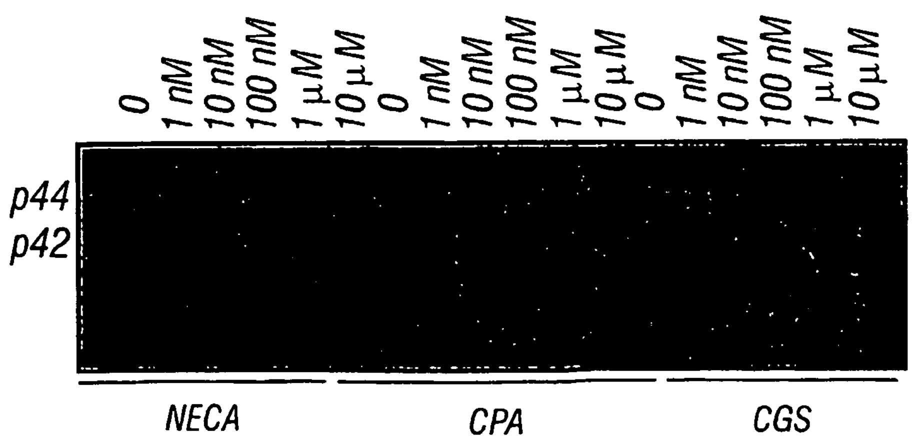


FIG. 4C

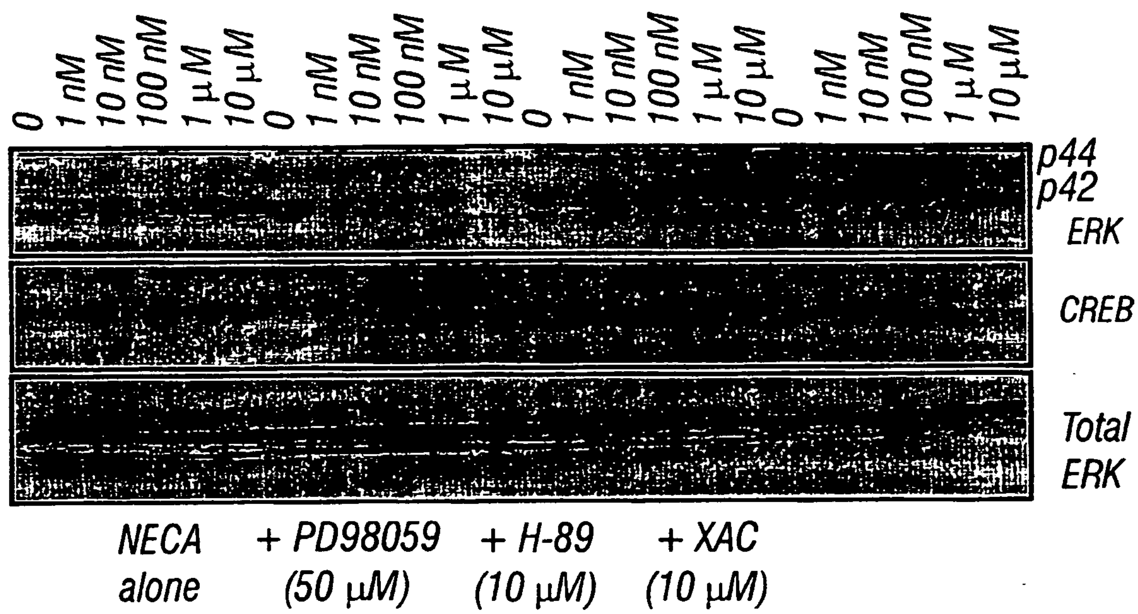

0
1 nM
10 nM
100 nM
1 μM
10 μM
0
1 nM
10 nM
100 nM
1 μM
10 μM
0
1 nM
10 nM
100 nM
1 μM
10 μM
0
1 nM
10 nM
100 nM
1 μM
10 μM
p44
p42
ERK
CREB
Total ERK
NECA alone
+ PD98059 (50 μM)
+ H-89 (10 μM)
+ XAC (10 μM)

FIG. 5

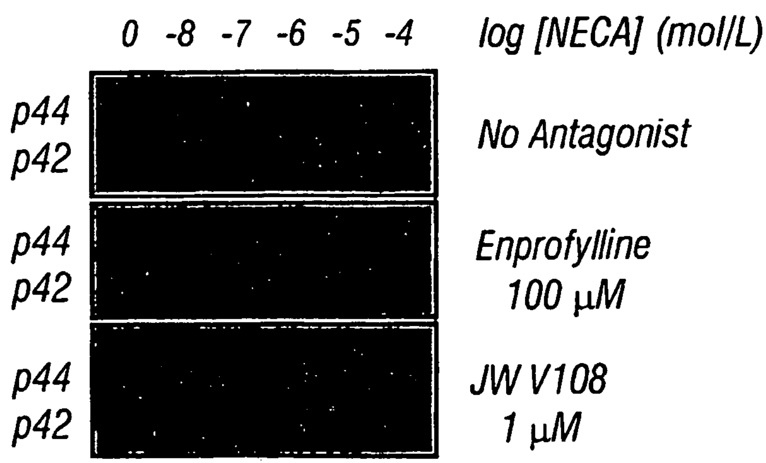

0 -8 -7 -6 -5 -4
log [NECA] (mol/L)
p44
p42
No Antagonist
p44
p42
Enprofylline
100 μM
p44
p42
JW V108
1 μM

FIG. 6

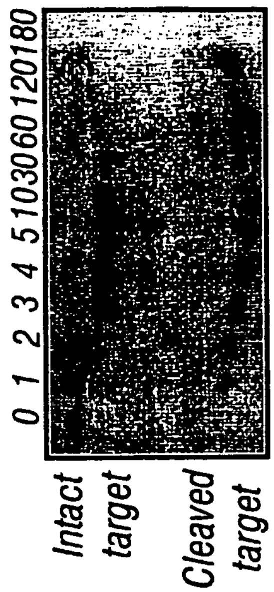


FIG. 7B

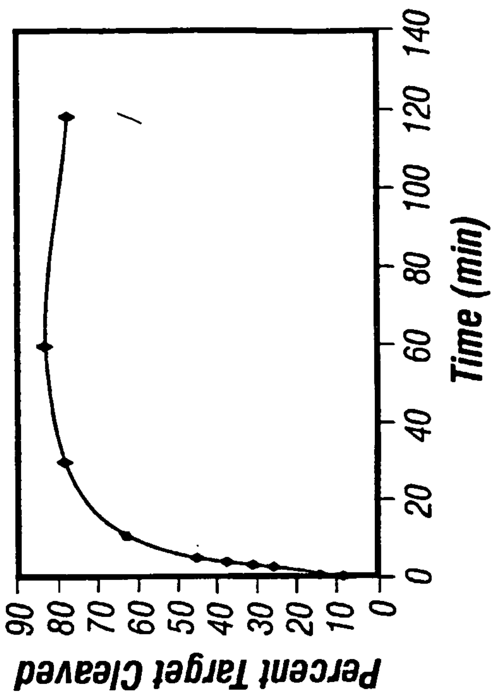


FIG. 7C

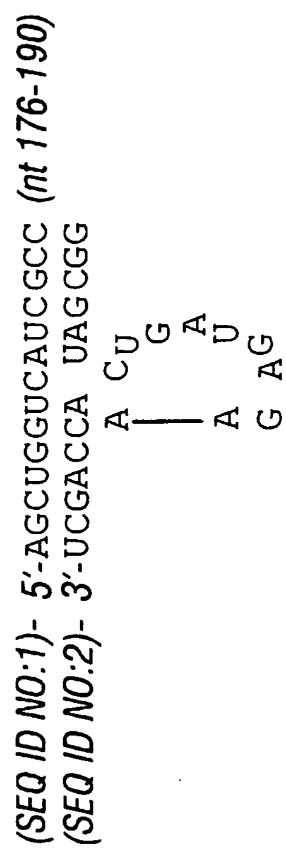
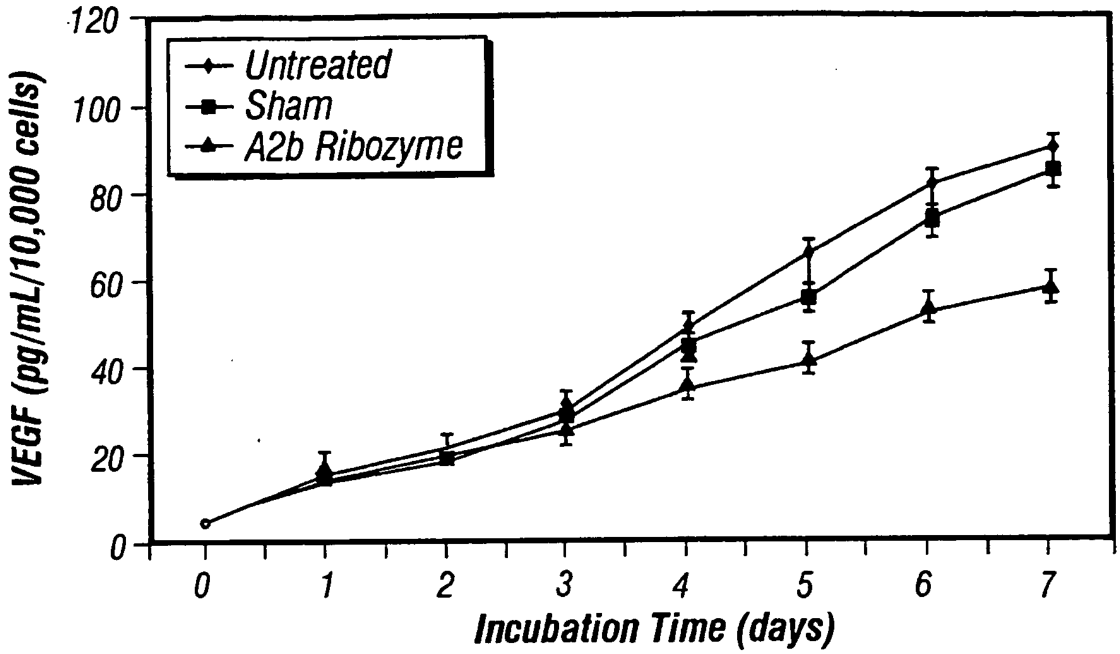
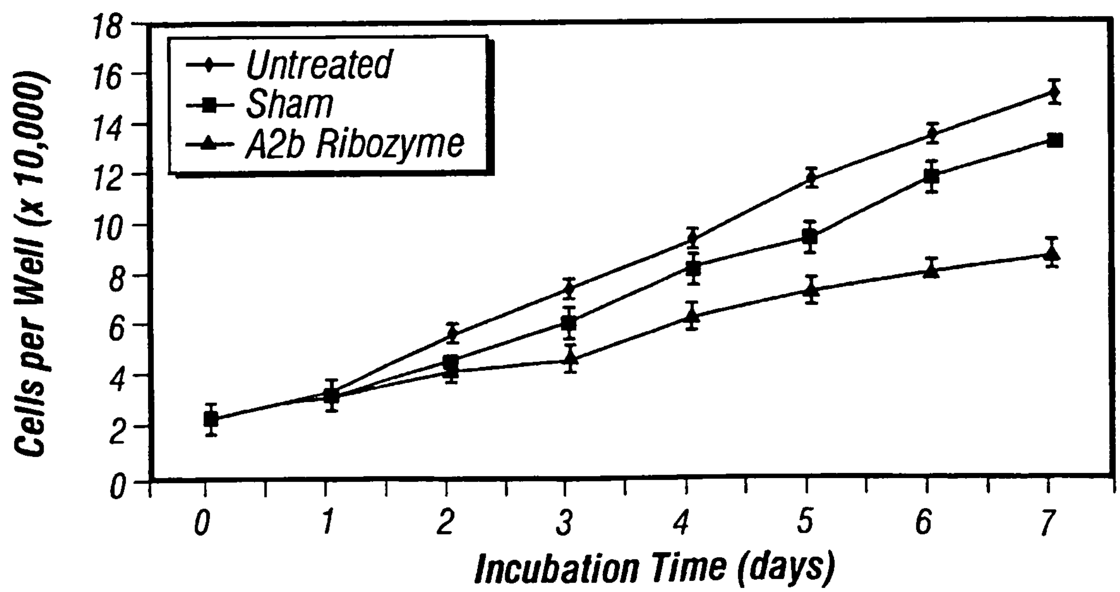


FIG. 7A



120
100
80
60
40
20
0
VEGF (pg/mL/10,000 cells)
Untreated
Sham
A2b Ribozyme
0 1 2 3 4 5 6 7
Incubation Time (days)

FIG. 8A



18
16
14
12
10
8
6
4
2
0
Cells per Well (x 10,000)
Untreated
Sham
A2b Ribozyme
0 1 2 3 4 5 6 7
Incubation Time (days)

FIG. 8B

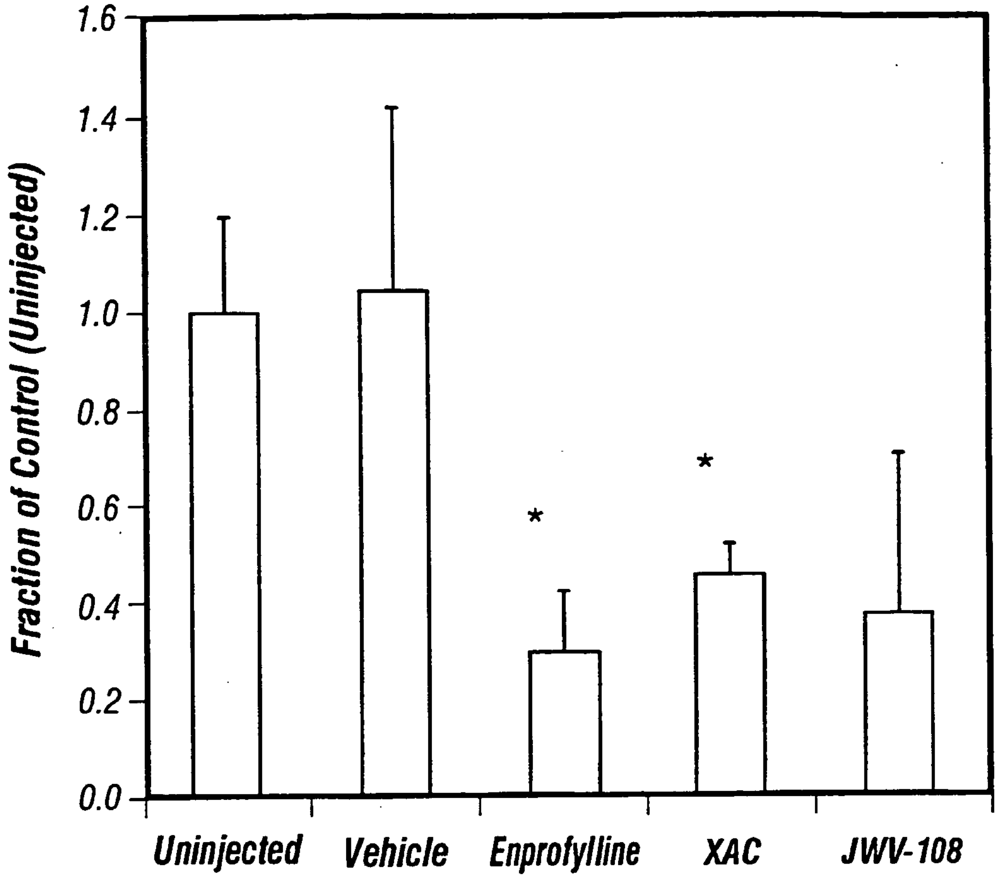

Fraction of Control (Uninjected)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Uninjected
Vehicle
Enprofylline
XAC
JWV-108

FIG. 9

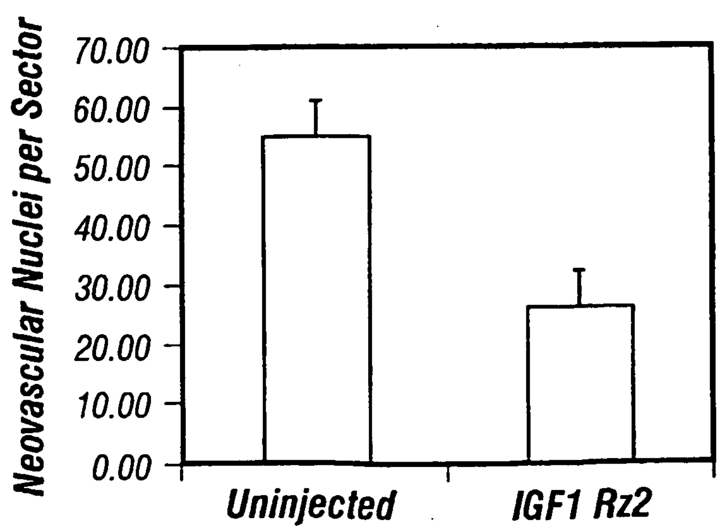

Neovascular Nuclei per Sector
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Uninjected
IGF1 Rz2

FIG. 10

Consensus Hairpin ribozyme:

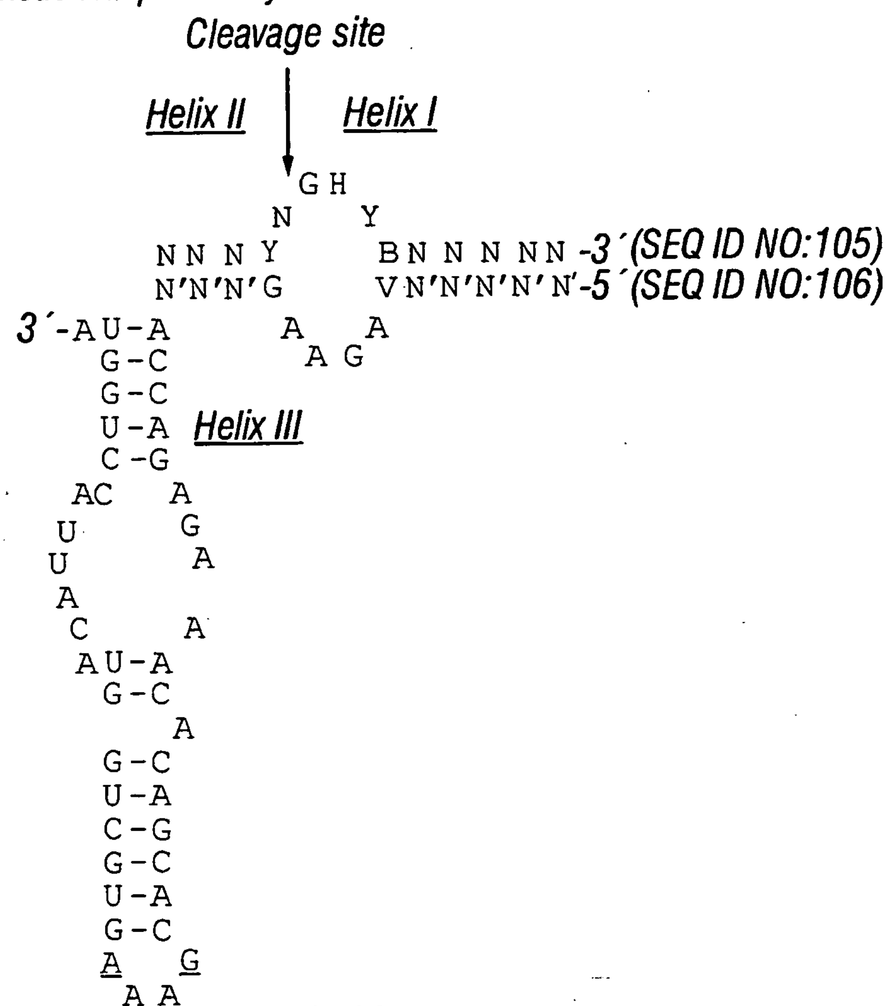


FIG. 11

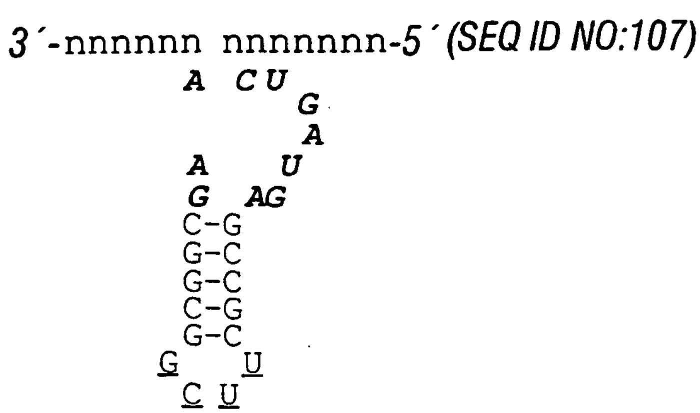


FIG. 12

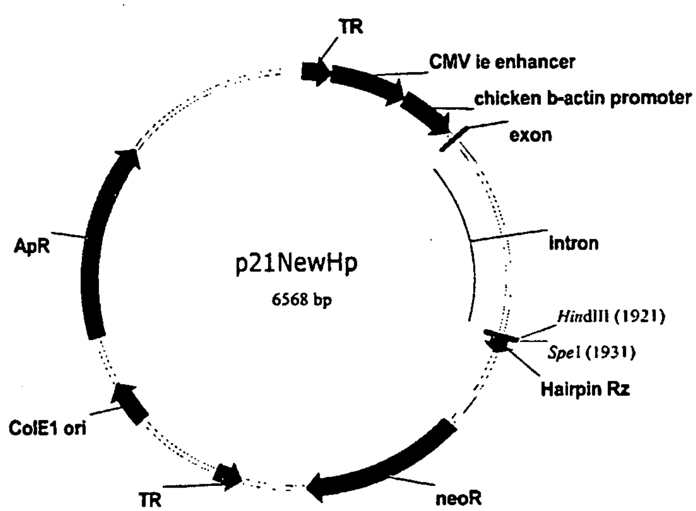

TR
CMV ie enhancer
chicken b-actin promoter
exon
intron
HindIII (1921)
SpeI (1931)
Hairpin Rz
neoR
TR
ColE1 ori
ApR
p21NewHp
6568 bp

FIG. 13

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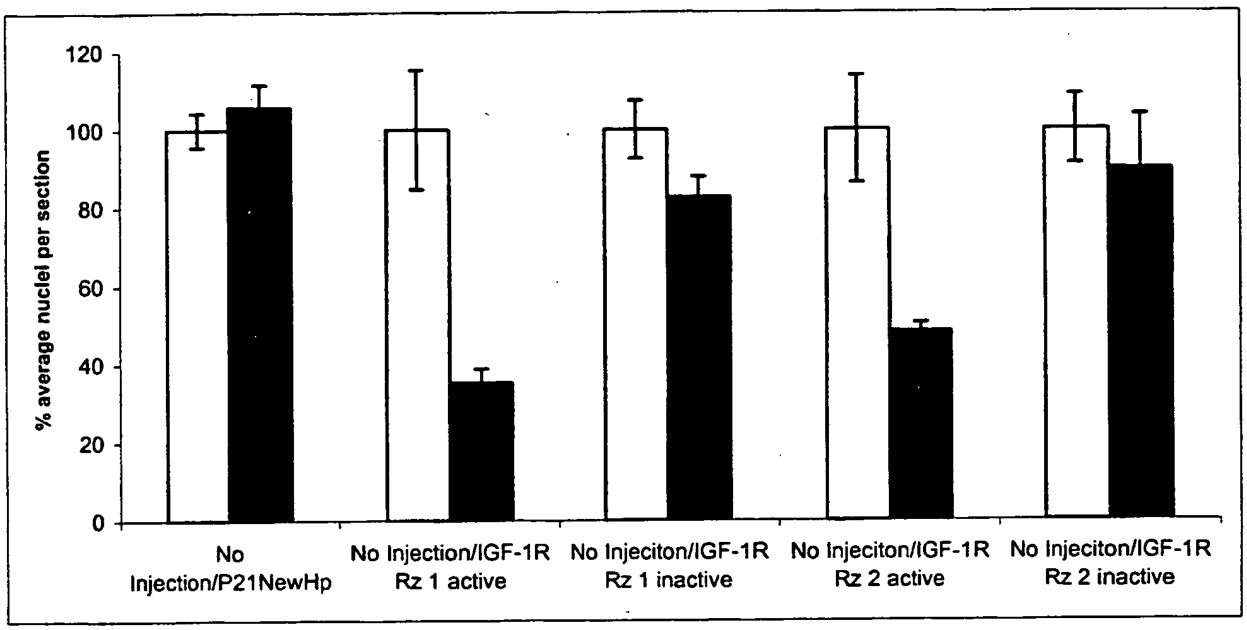

% average nuclei per section
120
100
80
60
40
20
0
No Injection/P21NewHp
No Injection/IGF-1R Rz 1 active
No Injeciton/IGF-1R Rz 1 inactive
No Injeciton/IGF-1R Rz 2 active
No Injeciton/IGF-1R Rz 2 inactive

FIG. 14

FIG. 15A

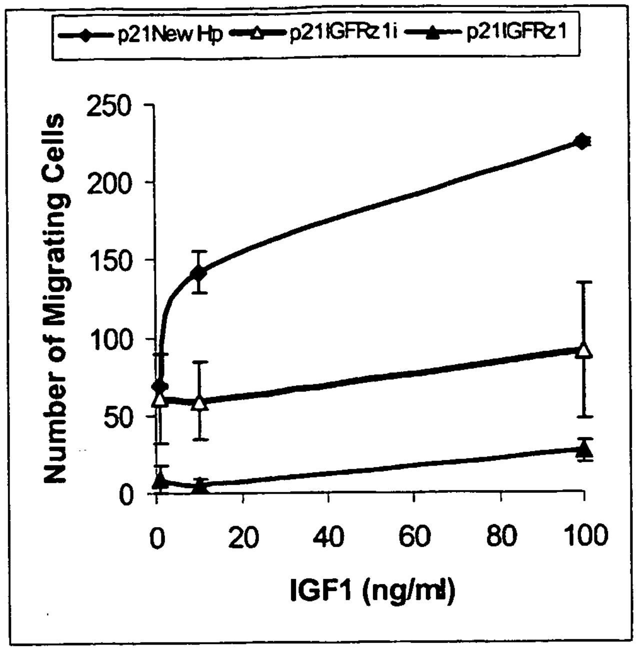
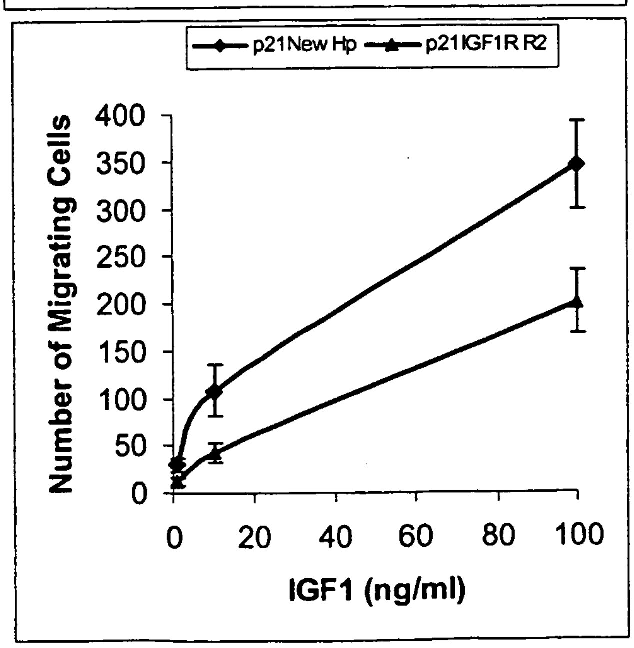


FIG. 15B



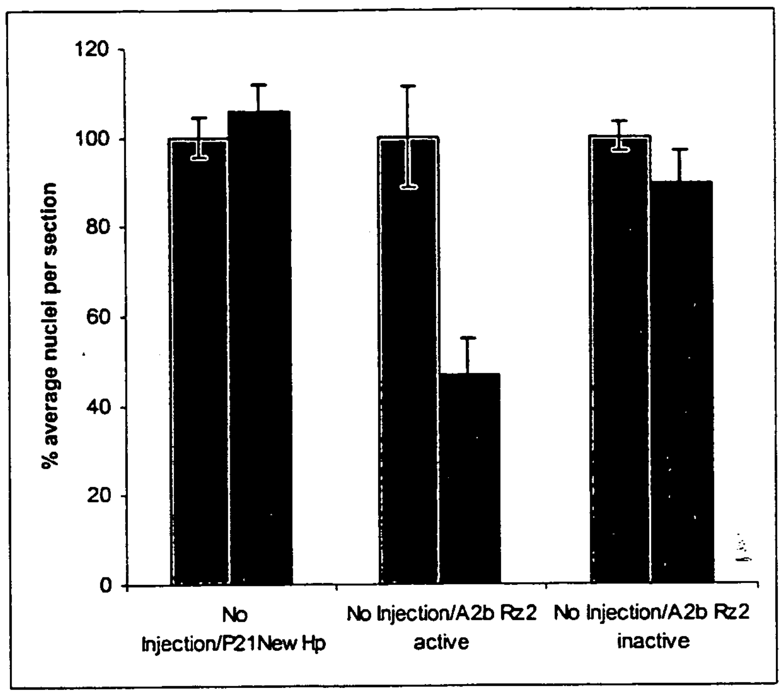

120
100
80
60
40
20
0
% average nuclei per section
No Injection/P21New Hp
No Injection/A2b Rz2 active
No Injection/A2b Rz2 inactive

FIG. 16